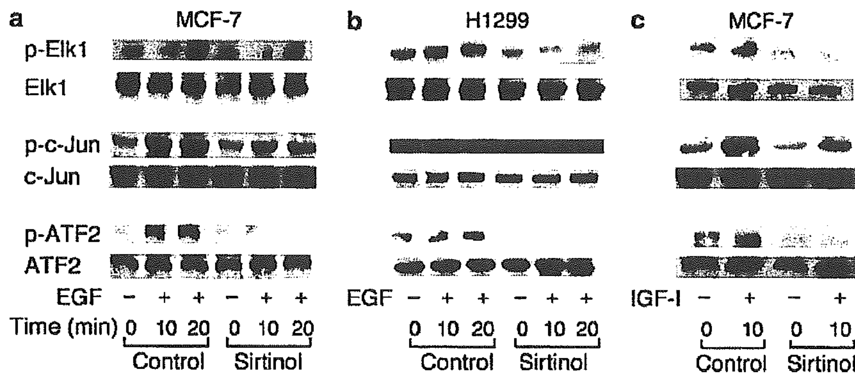

Figure 9 Growth factor-stimulated phosphorylation of Elk1, c-Jun and ATF2. At 10 days after the addition of Sirtinol (100 μM), following overnight serum starvation, the cells were exposed to EGF (50 ng/ml) for 10 or 20 min (**a**, **b**) or to IGF-I (100 ng/ml) for 10 min (**c**). In untreated (Control) MCF-7 and H1299 cells, marked phosphorylation of Elk1, c-Jun and ATF2 was induced by EGF and IGF-I. In Sirtinol-treated MCF-7 and H1299 cells, both basal (unstimulated) and EGF- or IGF-I-stimulated phosphorylation of Elk1, c-Jun and ATF2 were decreased compared with untreated cells.

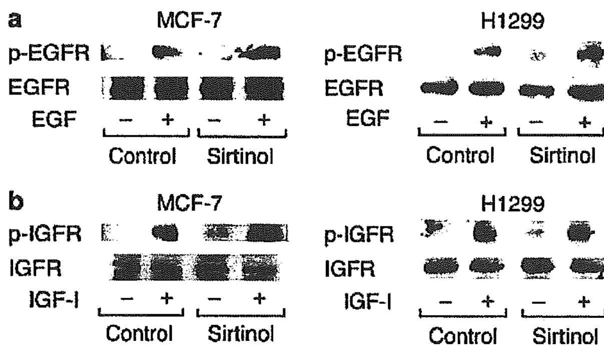

Figure 10 Growth factor-stimulated phosphorylation of EGF receptor and IGF-I receptor in Sirtinol-treated cells. At 10 days after the addition of Sirtinol (100 μM), following overnight serum starvation, the cells were exposed to EGF (50 ng/ml) (**a**) or IGF-I (100 ng/ml) for 2 min (**b**). There was no difference in tyrosine phosphorylation and protein expression of EGF receptor (EGFR) and IGF-I receptor (IGFR) between Sirtinol-treated and untreated (Control) MCF-7 and H1299 cells.

In contrast to the Ras–MAPK pathway, EGF- or IGF-I-induced as well as basal (unstimulated) phosphorylation of Akt/PKB was not decreased in Sirtinol-treated senescent MCF-7 and H1299 cells compared with untreated cells at 10 days after Sirtinol addition (Figure 12). The expression of Akt/PKB was not altered by Sirtinol treatment, either.

Discussion

We found that Sirt1 inhibition by specific inhibitors, Sirtinol and Splitomicin, and siRNA caused senescence-like growth arrest in human cancer MCF-7 and H1299 cells, as judged by SA-β-gal staining, PAI-1 expression, BrdU incorporation, flattened and enlarged morphology of the cells and flow cytometric analysis (Figures 1–6). Sirtinol-induced senescence-like growth arrest was accompanied by attenuated responses to growth factors in terms of activation of Ras–MAPKs (Figures 8, 9 and 11). By contrast, phosphorylation (activation) of EGF receptor, IGF-I receptor and Akt/PKB by growth factors was not affected in Sirtinol-treated senescent MCF-7 and H1299 cells (Figures 10 and 12).

Consistent with impaired activation of MAPKs, EGF- and IGF-I-stimulated phosphorylation of downstream targets, Elk-1, c-Jun and ATF-2, was also reduced (Figure 9). A hallmark feature of senescent cells is unresponsiveness to mitogenic stimuli in terms of induction of c-*fos* as well as cell proliferation. Previous studies in senescent human diploid fibroblasts showed that induction of c-*fos* (Seshadri and Campisi, 1990) and activation of Elk-1 (Tresini *et al.*, 2001) and MEK–ERK (Torres *et al.*, 2003) in response to growth factors are impaired. Transcriptional activity of Elk-1 regulates the induction of c-*fos*, an immediate early gene. Thus, our results of attenuated activation of Ras–MEK–ERK–Elk-1 in Sirtinol-treated senescent cancer cells are in agreement with previous findings in senescent human fibroblasts.

Our results indicate that the signaling defect in Sirtinol-treated cells is specific for MAPK pathways and that the PI3-K–Akt/PKB pathway is preserved. Ras is a key regulator of MAPK pathways (Lange-Carter and Johnson, 1994). However, Ras does not play a major role in activation of the PI3-K–Akt/PKB pathway (Sakaue *et al.*, 1995; Gnudi *et al.*, 1997; Klesse *et al.*, 1999). Our results showed that active, GTP-bound Ras was reduced in Sirtinol-treated cancer cells compared with untreated cells (Figure 11a). The present data, therefore, suggest that reduced activation of Ras might be involved in a specific attenuation in MAPK pathways in Sirtinol-treated senescent MCF-7 and H1299 cells.

Growth factor-initiated mitogenic signals are conveyed mainly by two major signaling cascades: Ras–ERK and PI3-K–Akt/PKB. Senescent cells remain viable and metabolically active, in spite of irreversible loss of replication capability (Roninson, 2003; Shay and Roninson, 2004). One can reasonably speculate, therefore, that the preserved PI3-K–Akt/PKB pathway might

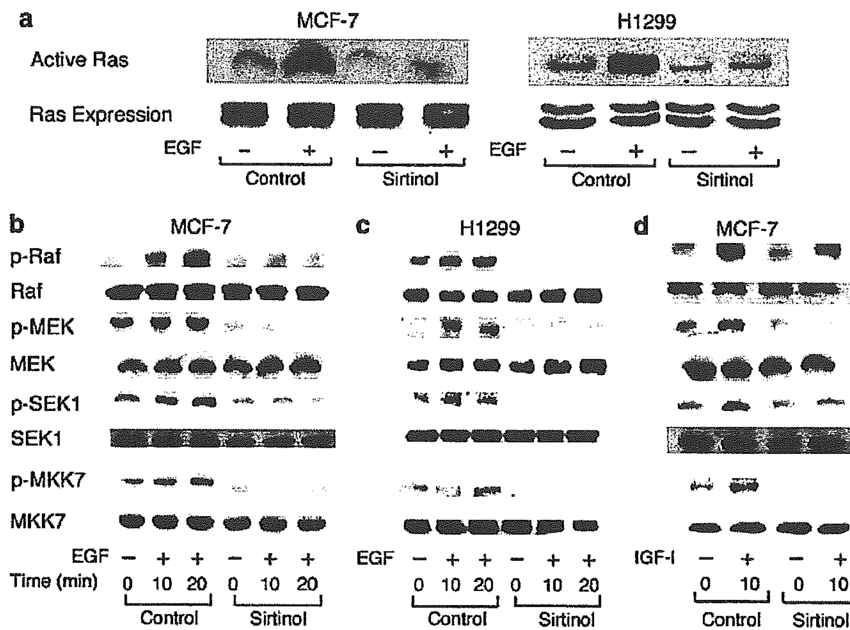

Figure 11 Activation status of Ras and its downstream signaling molecules in Sirtinol-treated cells. At 10 days after the addition of Sirtinol (100 μM), following overnight serum starvation, the cells were exposed to EGF (50 ng/ml) for 10 or 20 min, or IGF-I (100 ng/ml) for 10 min. (**a**) Active Ras was evaluated as described in Materials and methods. In untreated (Control) MCF-7 and H1299 cells, active Ras was markedly increased by EGF treatment for 20 min. In Sirtinol-treated MCF-7 and H1299 cells, basal (unstimulated) level of active Ras was decreased compared with untreated cells, and EGF failed to increase active Ras. (**b–d**) In untreated (Control) MCF-7 and H1299 cells, EGF and IGF-I induced robust phosphorylation of Raf, MEK, SEK1/MKK4 and MKK7. However, in Sirtinol-treated MCF-7 and H1299 cells, both basal (unstimulated) and EGF- or IGF-I-stimulated phosphorylation of these molecules were decreased compared with untreated (Control) cells.

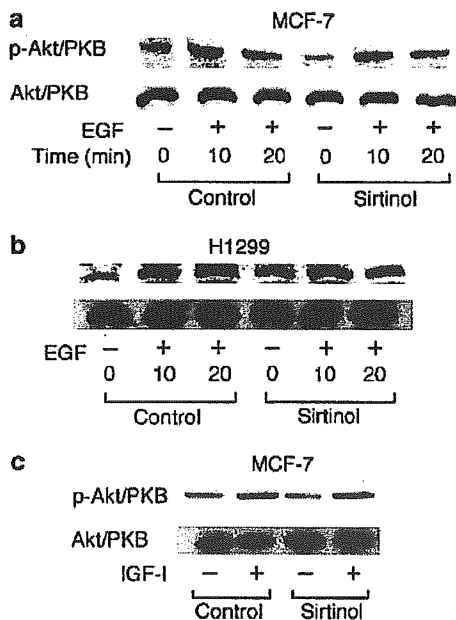

Figure 12 Growth factor-stimulated Akt/PKB phosphorylation in Sirtinol-treated cells. At 10 days after the addition of Sirtinol (100 μM), following overnight serum starvation, the cells were exposed to EGF (50 ng/ml) for 10 or 20 min (**a**, **b**) or to IGF-I (100 ng/ml) for 10 min (**c**). No difference was found in basal (unstimulated) and EGF- or IGF-I-stimulated phosphorylation of Akt/PKB between Sirtinol-treated and untreated (Control) MCF-7 and H1299 cells.

contribute to cell viability and metabolic activities in Sirtinol-treated cells, because the PI3-K–Akt/PKB pathway plays critical roles in cell survival and regulation of metabolism.

p53, Rb and cyclin-dependent kinase inhibitors such as p16, p21 and p27 have been recognized as key mediators of cellular senescence (Serrano *et al.*, 1997; Collado *et al.*, 2000; Alexander and Hinds, 2001; Beausejour *et al.*, 2003; Jirawatnotai *et al.*, 2003; Mallette *et al.*, 2004). Our results showed that hypophosphorylation of Rb and increased expression of p27 were associated with Sirtinol-induced senescence-like growth arrest in MCF-7 and H1299 cells (Figure 7). In addition to regulation at transcriptional level, increased p27 expression may result from reduced protein degradation through a ubiquitin–proteasome system (Carrano *et al.*, 1999). Since Sirt1 is an HDAC, it is possible that Sirt1 inhibition may directly modulate p27 transcription. However, our finding of unaltered p27 mRNA in Sirtinol-treated cells (Supplementary Figure 2) suggests that decreased protein degradation of p27, rather than increased transcription, may contribute to increased p27 protein expression in Sirtinol-treated cells. Thus, our data argue against a direct effect of Sirtinol on transcription of p27.

On the other hand, we did not find increased expression of p53, p21 and p16 in Sirtinol-treated MCF-7 and H1299 cells. It is important to note, however, that previous studies showed that premature senescence can be readily induced independent of p53,

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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References

Alexander K, Hinds PW. (2001). *Mol Cell Biol* **21**: 3616–3631.

Aviv H, Khan MY, Skurnick J, Okuda K, Kimura M, Gardner J *et al.* (2001). *Atherosclerosis* **159**: 281–287.

Beausejour CM, Krtolica A, Galimi F, Narita M, Lowe SW, Yaswen P *et al.* (2003). *EMBO J* **22**: 4212–4222.
Bedalov A, Gatbonton T, Irvine WP, Gottschling DE, Simon JA. (2001). *Proc Natl Acad Sci USA* **98**: 15113–15118.
Ben-Porath I, Weinberg RA. (2004). *J Clin Invest* **113**: 8–13.
Blander G, Guarente L. (2004). *Annu Rev Biochem* **73**: 417–435.
Brunet A, Sweeney LB, Sturgill JF, Chua KF, Greer PL, Lin Y *et al.* (2004). *Science* **303**: 2011–2015.
Carrano AC, Eytan E, Hershko A, Pagano M. (1999). *Nat Cell Biol* **1**: 193–199.
Chang BD, Broude EV, Dokmanovic M, Zhu H, Ruth A, Xuan Y *et al.* (1999a). *Cancer Res* **59**: 3761–3767.
Chang BD, Xuan Y, Broude EV, Zhu H, Schott B, Fang J *et al.* (1999b). *Oncogene* **18**: 4808–4818.
Cheng HL, Mostoslavsky R, Saito S, Manis JP, Gu Y, Patel P *et al.* (2003). *Proc Natl Acad Sci USA* **100**: 10794–10799.
Christov KT, Shilkaitis AL, Kim ES, Steele VE, Lubet RA. (2003). *Eur J Cancer* **39**: 230–239.
Cohen HY, Lavu S, Bitterman KJ, Hekking B, Imahiyerobo TA, Miller C *et al.* (2004). *Mol Cell* **13**: 627–638.
Collado M, Medema RH, Garcia-Cao I, Dubuisson ML, Barradas M, Glassford J *et al.* (2000). *J Biol Chem* **275**: 21960–21968.
Daitoku H, Hatta M, Matsuzaki H, Aratani S, Ohshima T, Miyagishi M *et al.* (2004). *Proc Natl Acad Sci USA* **101**: 10042–10047.
Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C *et al.* (1995). *Proc Natl Acad Sci USA* **92**: 9363–9367.
Elegbede JA, Hayes K, Schell K, Oberley TD, Verma AK. (2002). *Life Sci* **71**: 421–436.
Elmore LW, Rehder CW, Di X, McChesney PA, Jackson-Cook CK, Gewirtz DA *et al.* (2002). *J Biol Chem* **277**: 35509–35515.
Fujita N, Sato S, Katayama K, Tsuruo T. (2002). *J Biol Chem* **277**: 28706–28713.
Gnudi L, Frevert EU, Houseknecht KL, Erhardt P, Kahn BB. (1997). *Mol Endocrinol* **11**: 67–76.
Goldstein S, Moerman EJ, Fujii S, Sobel BE. (1994). *J Cell Physiol* **161**: 571–579.
Grozinger CM, Chao ED, Blackwell HE, Moazed D, Schreiber SL. (2001). *J Biol Chem* **276**: 38837–38843.
Han Z, Wei W, Dunaway S, Darnowski JW, Calabresi P, Sedivy J *et al.* (2002). *J Biol Chem* **277**: 17154–17160.
Imai S, Armstrong CM, Kaeberlein M, Guarente L. (2000). *Nature* **403**: 795–800.
Jirawatnotai S, Moons DS, Stocco CO, Franks R, Hales DB, Gibori G *et al.* (2003). *J Biol Chem* **278**: 17021–17027.
Kahlem P, Dorken B, Schmitt CA. (2004). *J Clin Invest* **113**: 169–174.
Kelly WK, Richon VM, O'Connor O, Curley T, MacGregor-Curtelli B, Tong W *et al.* (2003). *Clin Cancer Res* **9**: 3578–3588.
Kim JH, Lee GE, Kim SW, Chung IK. (2003). *Biochem J* **373**: 523–529.
Klesse LJ, Meyers KA, Marshall CJ, Parada LF. (1999). *Oncogene* **18**: 2055–2068.
Landry J, Sutton A, Tafrov ST, Heller RC, Stebbins J, Pillus L *et al.* (2000). *Proc Natl Acad Sci USA* **97**: 5807–5811.
Lange-Carter CA, Johnson GL. (1994). *Science* **265**: 1458–1461.
Langley E, Pearson M, Faretta M, Bauer UM, Frye RA, Minucci S *et al.* (2002). *EMBO J* **21**: 2383–2396.
Luo J, Nikolaev AY, Imai S, Chen D, Su F, Shiloh A *et al.* (2001). *Cell* **107**: 137–148.
Mallette FA, Goumard S, Gaumont-Leclerc MF, Moiseeva O, Ferbeyre G. (2004). *Oncogene* **23**: 91–99.
Mathon NF, Lloyd AC. (2001). *Nat Rev Cancer* **1**: 203–213.
McLaughlin F, La Thangue NB. (2004). *Curr Drug Targets Inflamm Allergy* **3**: 213–219.
Motta MC, Divecha N, Lemieux M, Kamel C, Chen D, Gu W *et al.* (2004). *Cell* **116**: 551–563.
Munro J, Barr NI, Ireland H, Morrison V, Parkinson EK. (2004). *Exp Cell Res* **295**: 525–538.
Pelicci PG. (2004). *J Clin Invest* **113**: 4–7.
Picard F, Kurtev M, Chung N, Topark-Ngarm A, Senawong T, Machado De Oliveira R *et al.* (2004). *Nature* **429**: 771–776.
Piekarz R, Bates S. (2004). *Curr Pharm Des* **10**: 2289–2298.
Piekarz RL, Robey R, Sandor V, Bakke S, Wilson WH, Dahmoush L *et al.* (2001). *Blood* **98**: 2865–2868.
Roninson IB. (2002). *Drug Resist Updat* **5**: 204–208.
Roninson IB. (2003). *Cancer Res* **63**: 2705–2715.
Rosato RR, Grant S. (2004). *Expert Opin Investig Drugs* **13**: 21–38.
Sakaue H, Hara K, Noguchi T, Matozaki T, Kotani K, Ogawa W *et al.* (1995). *J Biol Chem* **270**: 11304–11309.
Sandor V, Bakke S, Robey RW, Kang MH, Blagosklonny MV, Bender J *et al.* (2002). *Clin Cancer Res* **8**: 718–728.
Schmitt CA, Fridman JS, Yang M, Lee S, Baranov E, Hoffman RM *et al.* (2002). *Cell* **109**: 335–346.
Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW. (1997). *Cell* **88**: 593–602.
Seshadri T, Campisi J. (1990). *Science* **247**: 205–209.
Sharpless NE, DePinho RA. (2004). *J Clin Invest* **113**: 160–168.
Shay JW, Roninson IB. (2004). *Oncogene* **23**: 2919–2933.
Sugita H, Fujimoto M, Yasukawa T, Shimizu N, Sugita M, Yasuhara S *et al.* (2005). *J Biol Chem* **280**: 14203–14211.
Takahashi T, Nowakowski RS, Caviness Jr VS. (1992). *J Neurocytol* **21**: 185–197.
te Poele RH, Okorokov AL, Jardine L, Cummings J, Joel SP. (2002). *Cancer Res* **62**: 1876–1883.
Torres C, Francis MK, Lorenzini A, Tresini M, Cristofalo VJ. (2003). *Exp Cell Res* **290**: 195–206.
Tresini M, Lorenzini A, Frisoni L, Allen RG, Cristofalo VJ. (2001). *Exp Cell Res* **269**: 287–300.
van der Horst A, Tertoolen LG, de Vries-Smits LM, Frye RA, Medema RH, Burgering BM. (2004). *J Biol Chem* **279**: 28873–28879.
Vanhaecke T, Papeleu P, Elaut G, Rogiers V. (2004). *Curr Med Chem* **11**: 1629–1643.
Vaziri H, Dessain SK, Ng Eaton E, Imai SI, Frye RA, Pandita TK *et al.* (2001). *Cell* **107**: 149–159.
Vigushin DM, Coombes RC. (2004). *Curr Cancer Drug Targets* **4**: 205–218.
Wagner M, Hampel B, Bernhard D, Hala M, Zwerschke W, Jansen-Durr P. (2001). *Exp Gerontol* **36**: 1327–1347.
Wainwright LJ, Lasorella A, Iavarone A. (2001). *Proc Natl Acad Sci USA* **98**: 9396–9400.
Wang X, Tsao SW, Wong YC, Cheung AL. (2003). *Curr Cancer Drug Targets* **3**: 153–159.
Wright WE, Shay JW. (2001). *Curr Opin Genet Dev* **11**: 98–103.
Yasukawa T, Tokunaga E, Ota H, Sugita H, Martyn JA, Kaneki M. (2005). *J Biol Chem* **280**: 7511–7518.
Zhu WY, Jones CS, Kiss A, Matsukuma K, Amin S, De Luca LM. (1997). *Exp Cell Res* **234**: 293–299.

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

Wei Yu [a], Masahiro Akishita [b,*], Hang Xi [a], Kumiko Nagai [a], Noriko Sudoh [a], Hiroshi Hasegawa [a], Koichi Kozaki [a], Kenji Toba [a]

[a] *Department of Geriatric Medicine, Kyorin University School of Medicine, Tokyo, Japan*

[b] *Department of Geriatric Medicine, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan*

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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.
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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.

* Corresponding author. Tel.: +81 3 5800 8832; fax: +81 3 5800 8831.
E-mail address: akishita-tky@umin.ac.jp (M. Akishita).

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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.

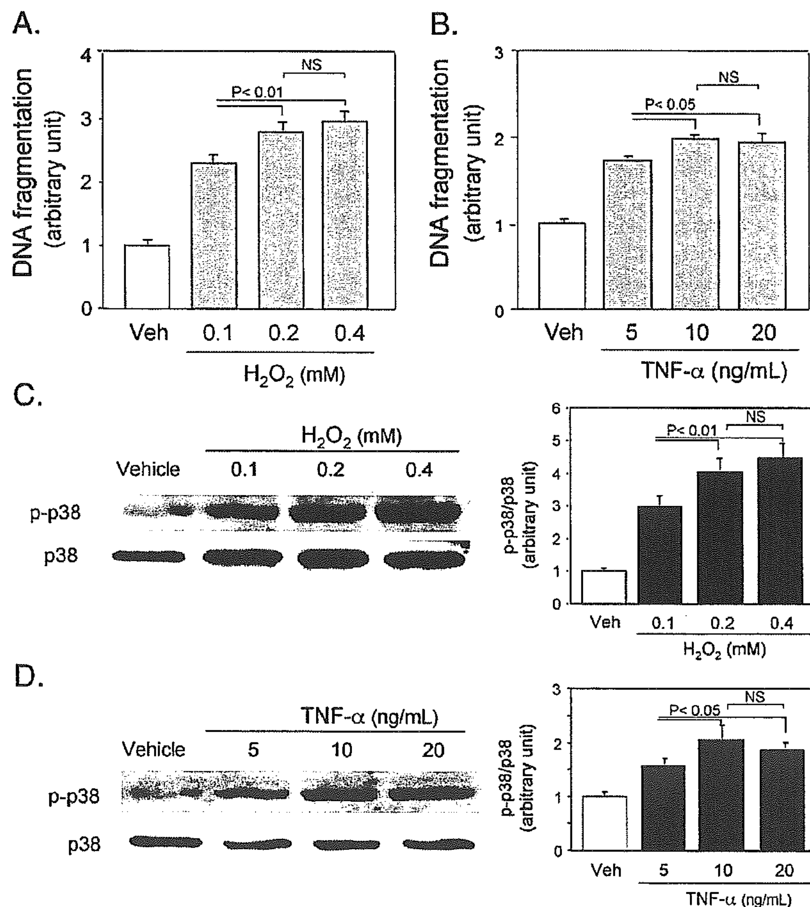

Fig. 1. Dose-dependent effects of H_2O_2 (A, C) and TNF-α (B, D) on EC apoptosis (A, B) and p38 MAP kinase activity (C, D). A and B, apoptosis was evaluated 24 h after H_2O_2 treatment (for 1.5 h) or 72 h after addition of TNF-α by means of DNA fragmentation ($n=3$). C and D, the activity of p38 MAP 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

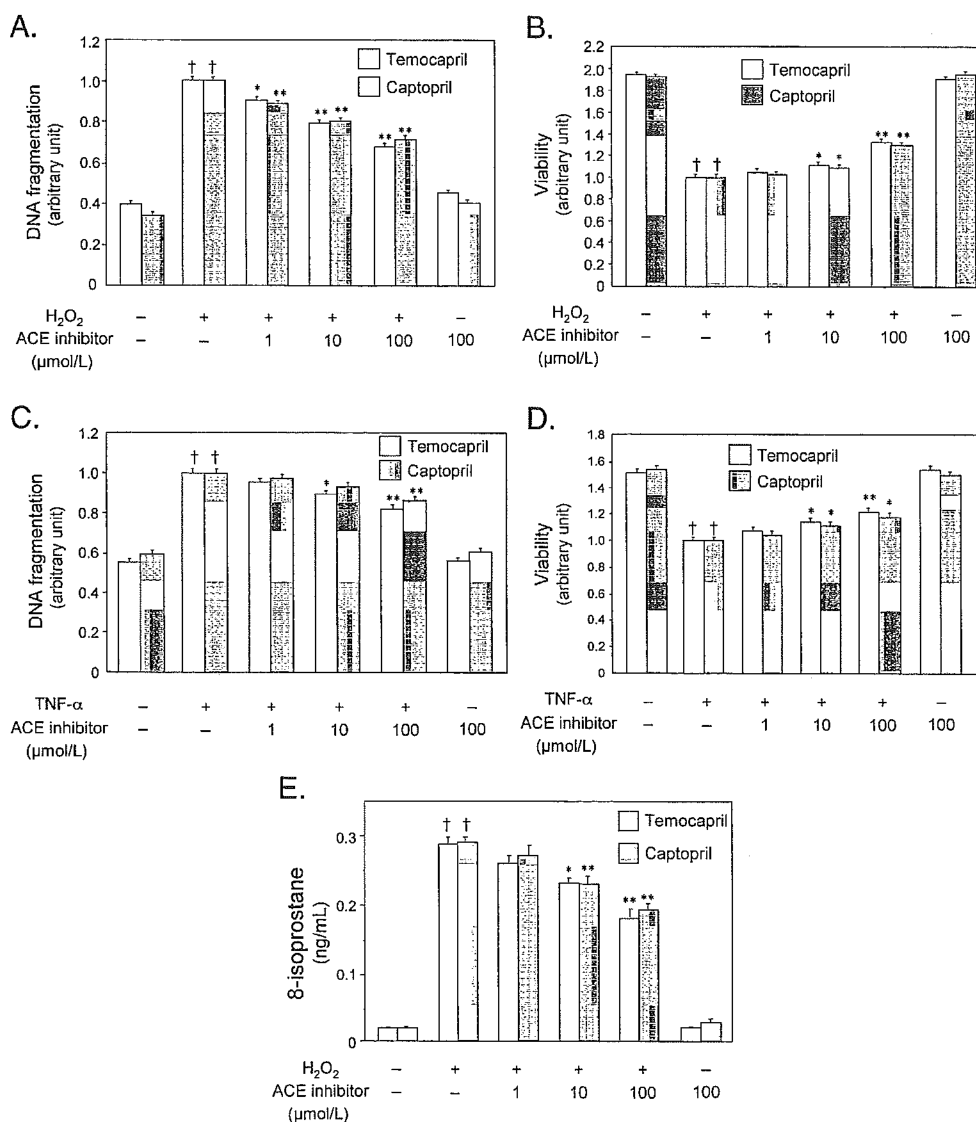

Fig. 2. Effects of ACE inhibitors on H_2O_2-induced (A, B) and TNF-α-induced (C, D) EC apoptosis and the effects of ACE inhibitors on H_2O_2-induced 8-isoprostne formation (E). Temocapril, captopril or their vehicle was added to the culture medium 24 h before H_2O_2 or TNF-α treatment until assay. Apoptosis (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

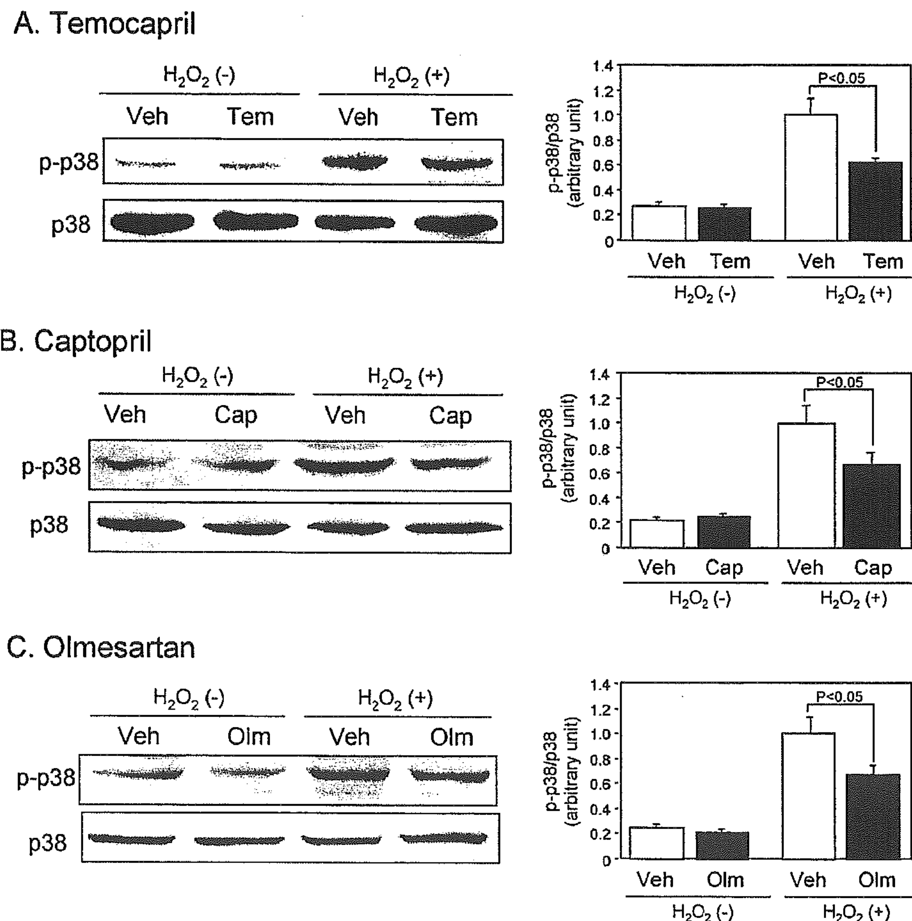

Fig. 3. Effects of temocapril (A), captopril (B) and olmesartan (C) on p38 MAP kinase activity at 30 min after exposure to H_2O_2. Temocapril (100 μmol/l), captopril (100 μmol/l), olmesartan (10 μmol/l) or its vehicle was added to the culture medium 24 h before H_2O_2 treatment until assay. Right panels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

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.

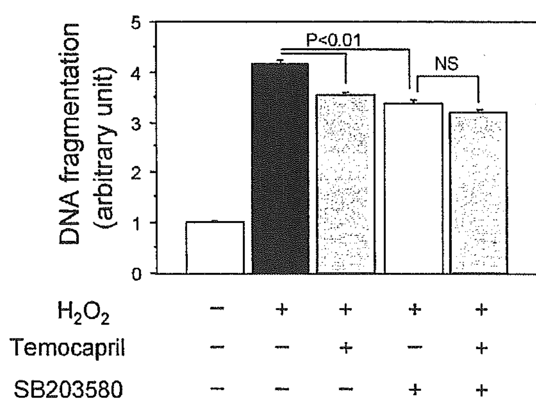

Fig. 4. Effects of temocapril and SB203580 on H_2O_2-induced EC apoptosis. Temocapril (100 μmol/l) or its vehicle was added to the culture medium 24 h before H_2O_2 treatment until assay. SB203580 (10 μmol/l) or its vehicle was added to the culture medium for 1 h before H_2O_2 treatment. EC apoptosis 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.

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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References

[1] Ross R. Atherosclerosis—an inflammatory disease. N Engl J Med 1999;340:115–26.

[2] Griendling KK, Sorescu D, Lassegue B, Ushio-Fukai M. Modulation of protein kinase activity and gene expression by reactive oxygen species and their role in vascular physiology and pathophysiology. Arterioscler Thromb Vasc Biol 2000;20:2175–83.

[3] Zalba G, San Jose G, Moreno MU, et al. Oxidative stress in arterial hypertension: role of NAD(P)H oxidase. Hypertension 2001;38:1395–9.

[4] Sorescu D, Weiss D, Lassegue B, et al. Superoxide production and expression of nox family proteins in human atherosclerosis. Circulation 2002;105:1429–35.

[5] Spiekermann S, Landmesser U, Dikalov S, et al. Electron spin resonance characterization of vascular xanthine and NAD(P)H oxidase activity in patients with coronary artery disease: relation to endothelium-dependent vasodilation. Circulation 2003;107:1383–9.

[6] Rey FE, Li XC, Carretero OA, Garvin JL, Pagano PJ. Perivascular superoxide anion contributes to impairment of endothelium-dependent relaxation: role of gp91(phox). Circulation 2002;106:2497–502.

[7] Pratico D, Tangirala RK, Rader DJ, Rokach J, FitzGerald GA. Vitamin E suppresses isoprostane generation in vivo and reduces atherosclerosis in ApoE-deficient mice. Nat Med 1998;4:1189–92.

[8] Fennell JP, Brosnan MJ, Frater AJ, et al. Adenovirus-mediated overexpression of extracellular superoxide dismutase improves endothelial dysfunction in a rat model of hypertension. Gene Ther 2002;9:110–7.

[9] Unger T. Blood pressure lowering and renin–angiotensin system blockade. J Hypertens 2003;6:S3–7.

[10] Scribner AW, Loscalzo J, Napoli C. The effect of angiotensin-converting enzyme inhibition on endothelial function and oxidant stress. Eur J Pharmacol 2003;482:95–9.

[11] Akishita M, Shirakami G, Iwai M, et al. Angiotensin converting enzyme inhibitor restrains inflammation-induced vascular injury in mice. J Hypertens 2001;19:1083–8.

[12] Antony I, Lerebours G, Nitenberg A. Angiotensin-converting enzyme inhibition restores flow-dependent and cold pressor test-induced dilations in coronary arteries of hypertensive patients. Circulation 1996;94:3115–22.

[13] Warnholtz A, Nickenig G, Schulz E, et al. Increased NADH-oxidase-mediated superoxide production in the early stages of atherosclerosis: evidence for involvement of the renin–angiotensin system. Circulation 1999;99:2027–33.

[14] Choy JC, Granville DJ, Hunt DW, McManus BM. Endothelial cell apoptosis: biochemical characteristics and potential implications for atherosclerosis. J Mol Cell Cardiol 2001;33:1673–90.

[15] Dimmeler S, Haendeler J, Zeiher AM. Regulation of endothelial cell apoptosis in atherothrombosis. Curr Opin Lipidol 2002;13:531–6.

[16] Akishita M, Kozaki K, Eto M, et al. Estrogen attenuates endothelin-1 production by bovine endothelial cells via estrogen receptor. Biochem Biophys Res Commun 1998;251:17–21.

[17] Dimmeler S, Haendeler J, Nehls M, Zeiher AM. Suppression of apoptosis by nitric oxide via inhibition of interleukin-1beta-converting enzyme (ICE)-like and cysteine protease protein (CPP)-32-like proteases. J Exp Med 1997;185:601–7.

[18] Grethe S, Ares MP, Andersson T, Porn-Ares MI. p38 MAPK mediates TNF-induced apoptosis in endothelial cells via phosphorylation and downregulation of Bcl-x(L). Exp Cell Res 2004;298:632–42.

[19] Johnson GL, Lapadat R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science 2002;298:1911–2.

[20] Cross TG, Scheel-Toellner D, Henriquez NV, Deacon E, Salmon M, Lord JM. Serine/threonine protein kinases and apoptosis. Exp Cell Res 2000;256:34–41.

[21] Rueckschloss U, Quinn MT, Holtz J, Morawietz H. Dose-dependent regulation of NAD(P)H oxidase expression by angiotensin II in human endothelial cells: protective effect of angiotensin II type 1 receptor blockade in patients with coronary artery disease. Arterioscler Thromb Vasc Biol 2002;22:1845–51.

[22] Landmesser U, Cai H, Dikalov S, et al. Role of p47(phox) in vascular oxidative stress and hypertension caused by angiotensin II. Hypertension 2002;40:511–5.

[23] Higashi Y, Sasaki S, Nakagawa K, Matsuura H, Oshima T, Chayama K. Endothelial function and oxidative stress in renovascular hypertension. N Engl J Med 2002;346:1954–62.

[24] Varin R, Mulder P, Tamion F, et al. Improvement of endothelial function by chronic angiotensin-converting enzyme inhibition in heart

failure: role of nitric oxide, prostanoids, oxidant stress, and bradykinin. Circulation 2000;102:351–6.
[25] Onozato ML, Tojo A, Goto A, Fujita T, Wilcox CS. Oxidative stress and nitric oxide synthase in rat diabetic nephropathy: effects of ACEI and ARB. Kidney Int 2002;61:186–94.
[26] de Cavanagh EM, Piotrkowski B, Basso N, et al. Enalapril and losartan attenuate mitochondrial dysfunction in aged rats. FASEB J 2003;17:1096–8.
[27] Hornig B, Landmesser U, Kohler C, et al. Comparative effect of ace inhibition and angiotensin II type 1 receptor antagonism on bioavailability of nitric oxide in patients with coronary artery disease: role of superoxide dismutase. Circulation 2001;103:799–805.
[28] Kofler S, Nickel T, Weis M. Role of cytokines in cardiovascular diseases: a focus on endothelial responses to inflammation. Clin Sci (Lond) 2005;108:205–13.
[29] Shakibaei M, Schulze-Tanzil G, Takada Y, Aggarwal BB. Redox regulation of apoptosis by members of the TNF superfamily. Antioxid Redox Signal 2005;7:482–96.
[30] Griendling KK, Sorescu D, Lassegue B, Ushio-Fukai M. Modulation of protein kinase activity and gene expression by reactive oxygen species and their role in vascular physiology and pathophysiology. Arterioscler Thromb Vasc Biol 2000;20:2175–83.
[31] Huot J, Houle F, Rousseau S, Deschesnes RG, Shah GM, Landry J. SAPK2/p38-dependent F-actin reorganization regulates early membrane blebbing during stress-induced apoptosis. J Cell Biol 1998; 143:1361–73.
[32] Chen K, Vita JA, Berk BC, Keaney Jr JF. c-Jun N-terminal kinase activation by hydrogen peroxide in endothelial cells involves SRC-dependent epidermal growth factor receptor transactivation. J Biol Chem 2001;276:16045–50.
[33] Thomas SR, Chen K, Keaney Jr JF. Hydrogen peroxide activates endothelial nitric-oxide synthase through coordinated phosphorylation and dephosphorylation via a phosphoinositide 3-kinase-dependent signaling pathway. J Biol Chem 2002;277:6017–24.
[34] Shi Y, Gaestel M. In the cellular garden of forking paths: how p38 MAPKs signal for downstream assistance. Biol Chem 2002;383: 1519–36.
[35] Nakagami H, Morishita R, Yamamoto K, et al. Phosphorylation of p38 mitogen-activated protein kinase downstream of bax-caspase-3 pathway leads to cell death induced by high D-glucose in human endothelial cells. Diabetes 2001;50:1472–81.
[36] Takahashi M, Okazaki H, Ogata Y, Takeuchi K, Ikeda U, Shimada K. Lysophosphatidylcholine induces apoptosis in human endothelial cells through a p38-mitogen-activated protein kinase-dependent mechanism. Atherosclerosis 2002;161:387–94.

Original Article

Impact of Blood Pressure Variability on Cardiovascular Events in Elderly Patients with Hypertension

Masato ETO, Kenji TOBA, Masahiro AKISHITA, Koichi KOZAKI, Tokumitsu WATANABE, Seungbum KIM, Masayoshi HASHIMOTO, Junya AKO, Katsuya IIJIMA, Noriko SUDOH, Masao YOSHIZUMI, and Yasuyoshi OUCHI

Blood pressure variability is one of the characteristic features of hypertension in the elderly. However, its clinical significance remains to be determined. We therefore examined the impact of blood pressure variability on the development of cardiovascular events in elderly hypertensive patients. A total of 106 consecutive hypertensive patients aged more than 60 years old (mean age, 73.9±8.1 years old; male, 54%), all of whom underwent 24-h ambulatory blood pressure monitoring, were followed up (median, 34 months; range, 3–60 months). During the follow-up period, 39 cardiovascular events were observed, including 14 cases of cerebral infarction and 7 cases of acute myocardial infarction. The coefficient of variation (CV) of 24-h systolic blood pressure (SBP) values was used as an index of blood pressure variability. The patients showed a mean CV value of 10.6%, and were divided into two groups according to this mean value as a cut-off point: a high CV group (n=46) and a low CV group (n=60). Although baseline clinical characteristics were similar in the two groups, Kaplan-Meier plots for event-free survival revealed that the rate of cardiovascular events was significantly higher in high CV group than in low CV group (p<0.05). Cox's proportional hazards analysis showed that increased blood pressure variability (a high CV value of 24-h SBP) was an independent predictive variable for cardiovascular events. The CV value of daytime SBP and the SD value of both 24-h SBP and daytime SBP also had positive correlations with the onset of cardiovascular events. These results suggest that increased blood pressure variability may be an independent risk factor for cardiovascular events in elderly hypertensive patients. (*Hypertens Res* 2005; 28: 1–7)

***Key Words*: elderly hypertension, blood pressure variability, cardiovascular events, ambulatory blood pressure monitoring**

Introduction

Hypertension has been well established as a major predisposing factor for cardiovascular disease (*1*). The goal of treatment for hypertensive patients is not only to reduce blood pressure, but also to prevent cardiovascular events. The prevalence of hypertension increases with age (*2*), and elderly hypertensive patients are known to have some specific clinical features, such as isolated systolic hypertension (*3*), blood pressure variability (*4*, *5*), orthostatic hypotension (*6*, *7*) and postprandial hypotension (*8*).

Blood pressure variability is a characteristic feature of hypertension in the elderly (*4*, *5*). The arterial baroreflex

From the Department of Geriatric Medicine, Graduate School of Medicine, University of Tokyo, Tokyo, Japan.
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Address for Reprints: Yasuyoshi Ouchi, M.D., Ph.D., Department of Geriatric Medicine, Graduate School of Medicine, University of Tokyo, 7–3–1 Hongo, Bunkyo-ku, Tokyo 113–8655, Japan. E-mail: youchi-tky@umin.ac.jp
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Table 1. Baseline Clinical Characteristics

	Total (n=106)	Low CV group (n=60)	High CV group (n=46)	p value
Age (years old; mean±SD)	73.9±8.1	74.4±7.9	73.2±8.3	NS
(range)	(60–91)	(60–91)	(60–87)	
Sex (men) (n (%))	58 (54%)	36 (60%)	22 (48%)	NS
WHO class (n (%))				
I	31 (29%)	22 (37%)	9 (20%)	NS
II	22 (21%)	12 (20%)	10 (22%)	
III	53 (50%)	26 (43%)	27 (58%)	
Smoking (n (%))	53 (50%)	32 (53%)	21 (46%)	NS
Antihypertensive drug (n (%))				
ACE inhibitor	19 (18%)	10 (17%)	9 (20%)	NS
β-Blocker	7 (7%)	4 (7%)	3 (7%)	
Ca channel blocker	82 (77%)	48 (80%)	34 (74%)	
Diuretics	13 (12%)	8 (13%)	5 (11%)	
Complicaton (n (%))				
Hypercholesterolemia	33 (31%)	21 (35%)	12 (26%)	NS
Diabetes	36 (34%)	22 (37%)	14 (30%)	NS
Cerebrovascular disease	32 (30%)	19 (32%)	13 (28%)	NS
Coronary artery disease	19 (18%)	9 (15%)	10 (22%)	NS
Total cholesterol (mg/dl; mean±SEM)	189.5±12.2	180.5±13.3	209.1±11.4	NS
Creatinine (mg/dl; mean±SEM)	1.0±0.1	0.9±0.1	1.0±0.1	NS

CV, coefficient of variation; ACE, angiotensin converting enzyme.

plays a pivotal role in the neural regulation of blood pressure, and blood pressure variability is regulated by this compensatory reflex mechanism. Arterial baroreflex function is decreased in elderly individuals (*9*, *10*), and as a result, their blood pressure fluctuates (*11*). Although the mechanism of blood pressure variability in the elderly has been well elucidated, its clinical significance remains to be determined. In particular, there is little available information on the relationship between blood pressure variability and cardiovascular events in elderly hypertensive patients.

We hypothesized that blood pressure variability would be an independent risk factor for cardiovascular events in elderly patients with hypertension. To test this hypothesis, we investigated the outcome of elderly patients who underwent ambulatory blood pressure monitoring (ABPM). The results demonstrated that increased blood pressure variability is an independent predictive variable for cardiovascular events.

Methods

Patients

We recruited a total of 106 consecutive hypertensive patients, aged 60 years or older, who underwent 24-h ABPM at the University of Tokyo Hospital. The age, sex, smoking status, World Health Organization/International Society of Hypertension (WHO/ISH) classification, presence or absence of hypercholesterolemia and diabetes, history of cerebrovascular disease and history of coronary artery disease of each patient were investigated as baseline clinical characteristics according to their medical records. Hypertension was defined as an office systolic blood pressure (SBP) level above 140 mmHg and/or an office diastolic blood pressure (DBP) level above 90 mmHg on more than two occasions or the use of antihypertensive drugs. Smokers were defined as current smokers. Hypercholesterolemia was defined as a serum total cholesterol concentration above 220 mg/dl or the use of lipid-lowering drugs. Diabetes mellitus was defined as a fasting plasma glucose concentration above 140 mg/dl or use of antidiabetic medication. None showed severe renal failure (serum creatinine>2.0 mg/dl). Informed consent for this study was obtained from all patients.

Twenty-Four-Hour ABPM

Ambulatory blood pressure was recorded with a noninvasive automatic ABPM device (ABPM-630; Nippon Colin, Komaki, Japan) every 30 min for 24 h. The data used in this study were obtained by the oscillometric method. The accuracy of this device was previously described (*12*). Patients were not included in the study if their blood pressure could not be evaluated because of artifacts in more than 10% of the total measurements.

The mean values of 24-h, daytime (from 6:00 to 21:00) and nighttime (from 21:30 to 5:30) SBP and DBP were calculated for each patient. We calculated the coefficient of variation

Table 2. Profiles of 24 h, Daytime, Nighttime and Casual Blood Pressure

	Total (n=106)	Low CV group (n=60)	High CV group (n=46)
24 h blood pressure			
Systolic blood pressure (mmHg)	142.4±17.2	143.3±17.2	141.2±16.6
Diastolic blood pressure (mmHg)	78.1±10.3	79.2±10.6	76.8±9.9
CV of systolic blood pressure (%)	10.6±2.9	8.8±1.4	13.1±2.5*
Daytime blood pressure			
Systolic blood pressure	143.7±17.0	143.9±17.2	141.9±16.5
Diastolic blood pressure (mmHg)	79.2±10.4	79.7±10.9	78.6±9.9
Nighttime blood pressure (mmHg)			
Systolic blood pressure (mmHg)	140.1±20.3	142.0±18.5	137.7±20.7
Diastolic blood pressure (mmHg)	75.2±11.3	77.0±11.1	73.0±11.4
Casual blood pressure			
Systolic blood pressure (mmHg)	148.7±19.1	150.5±15.5	146.0±22.8
Diastolic blood pressure (mmHg)	81.4±11.6	82.0±10.0	81.0±13.0
Pulse pressure (mmHg)	67.3±16.6	69.1±16.0	64.8±17.3

Data are expressed as mean±SD. CV, coefficient of variation. *p<0.01.

(CV; CV=SD/mean value×100%) of 24-h SBP as an index of blood pressure variability. The CV values of daytime SBP and nighttime blood pressure as well as the SD values of 24-h SBP, daytime SBP and nighttime blood pressure were also calculated. Casual blood pressure was measured by the standard cuff method in the morning (9:00 to 12:00) when the ambulatory blood pressure was monitored.

To confirm the reproducibility, we compared the two subsequent measurements in 23 patients who underwent 24-h ABPM twice within 1 month. There were significant positive correlations between the two measurements of 3 parameters of 24-h blood pressure (24-h SBP, r=0.808, p<0.01; 24-h DBP, r=0.693, p<0.01; CV of 24-h SBP, r=0.564, p<0.01, n=23).

Follow-Up

Patients were followed up in the outpatient clinic of the hospital. Cardiovascular endpoints consisted of new onset of angina pectoris, acute myocardial infarction, coronary artery bypass graft surgery, percutaneous coronary intervention, sudden cardiac death, heart failure, cerebral infarction, cerebral hemorrhage, transient cerebral ischemic attack, acute aortic dissection and aortic graft replacement surgery for aortic aneurysm. Angina pectoris was diagnosed based on a history of chest pain and reversible ischemic change on electrocardiography during a spontaneous attack or exercise stress test. Acute myocardial infarction was diagnosed based on a history of chest pain, transient ST elevation on electrocardiography and increased serum myocardial enzyme concentrations. Sudden cardiac death was defined as a death that occurred within 1 h after the onset of symptoms. Heart failure was diagnosed based on clinical symptoms and signs and chest roentgenographic findings. Cerebral infarction and cerebral hemorrhage were diagnosed based on focal neurological deficits and brain computed tomographic findings. Transient cerebral ischemic attack was diagnosed based on focal neurological deficits that disappeared completely less than 24 h after the onset. Acute aortic dissection was diagnosed based on a history of chest, back and/or abdominal pain and thoracic and abdominal computed tomographic findings.

Data Analysis

To explore the clinical significance of blood pressure variability on cardiovascular events, we divided the patients into two groups: a high CV group and a low CV group, using the mean CV value of 24-h SBP (10.6%) as a cut-off point and compared the two groups in terms of baseline clinical characteristics, blood pressure profiles and the incidence of cardiovascular events. In addition, we divided the patients into two groups according to the mean values of CV of daytime and nighttime SBP and SD of 24-h, daytime and nighttime SBP and analyzed the data for each group. Data are expressed as the mean±SD. Categorical variables were compared by χ^2 test. Continuous variables were compared by Student's t-test. Kaplan-Meier curves were plotted for event free survival and compared by log rank test. Finally, Cox's proportional hazards analysis was performed to examine the relative risk for cardiovascular events using age, sex, WHO/ISH class, smoking, hypercholesterolemia, diabetes, history of cerebrovascular disease, history of coronary artery disease, mean 24-h blood pressure, mean daytime blood pressure, mean nighttime blood pressure, casual blood pressure, pulse pressure and CV (or SD) of SBP as variables. A value of p<0.05 was considered to be significant.

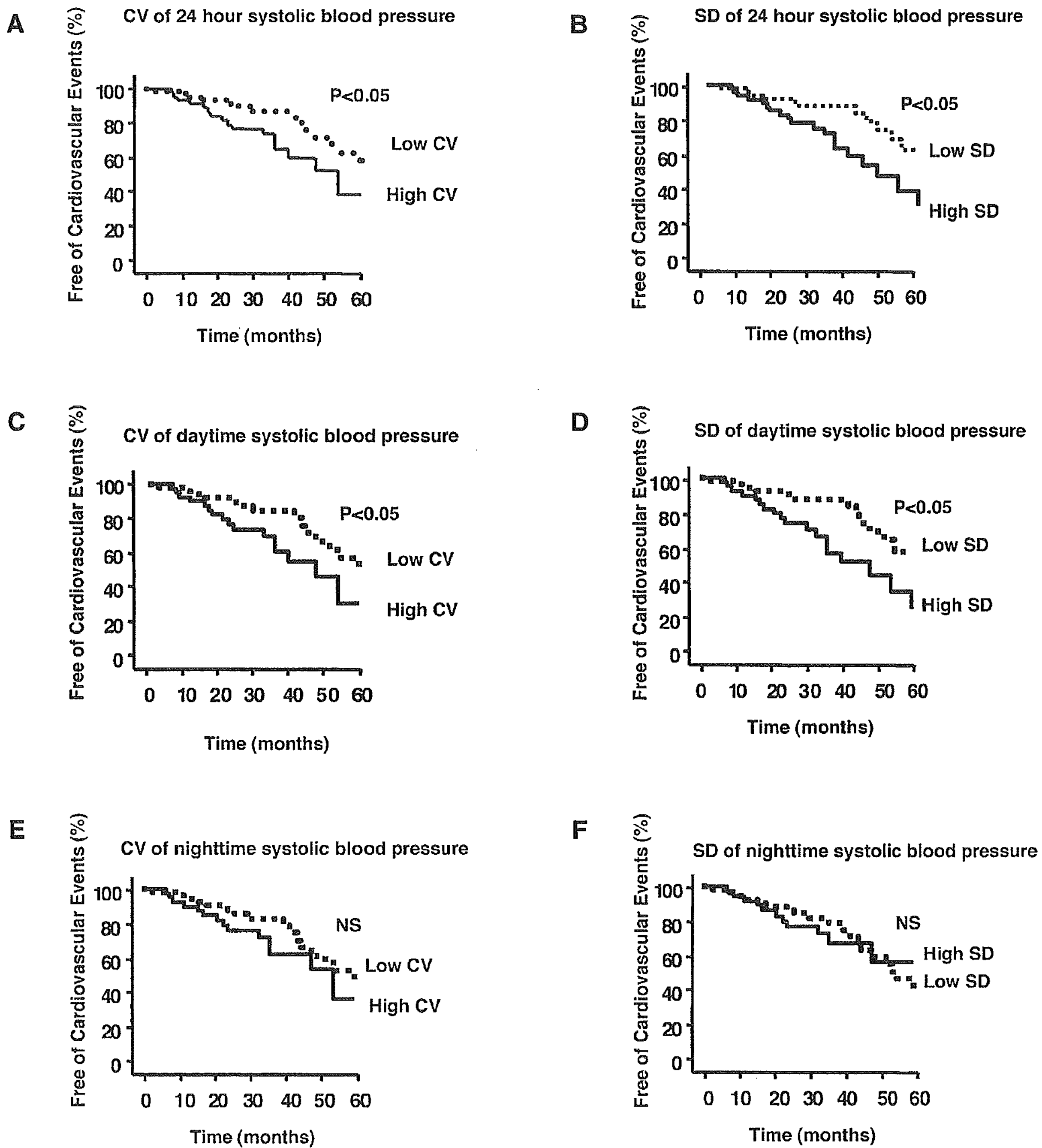

Fig. 1. *Cumulative event-free rates of cardiovascular events. Patients were divided into two groups according to the mean values of the CV of 24-h blood pressure (A), daytime blood pressure (C) or nighttime blood pressure (E), or those of the SD of 24-h blood pressure (B), daytime blood pressure (D) or nighttime blood pressure (F). CV, coefficient of variation.*

Results

The baseline clinical characteristics are shown in Table 1. All patients were treated with one or two antihypertensive drugs. Calcium channel blockers were used in 77% of the patients. ACE inhibitors, β-blockers and diuretics were used in 18%, 7% and 12% of the patients, respectively (Table 1). The

Table 3. Relative Risk of Cardiovascuiar Events

	Relative risk	95% CI
A		
Sex (male)	3.28	1.22–8.81*
24-h SBP (≥150 mmHg)	5.17	2.03–13.1**
CV of 24-h SBP (≥10.6%)	3.58	1.63–7.85*
B		
History of coronary artery disease	4.88	1.41–16.9*
24-h SBP (≥150 mmHg)	6.57	2.24–24.9*
SD of 24-h SBP (≥15.0 mmHg)	3.26	1.25–8.52*
C		
Sex (male)	3.22	1.14–9.09*
History of coronary artery disease	5.00	1.38–18.1*
24-h SBP (≥150 mmHg)	7.46	2.37–30.5*
CV of daytime SBP (≥11.4%)	3.72	1.08–15.1*
D		
History of coronary artery disease	4.94	1.41–18.1*
24-h SBP (≥150 mmHg)	6.63	2.23–25.8*
SD of daytime SBP (≥16.4 mmHg)	3.72	1.06–8.00*

Clinical characteristics, mean values of 24-h, daytime, nighttime and casual blood pressure, pulse pressure and SD of daytime, nighttime SBP are used as variables. *$p<0.05$, **$p<0.01$. CI, confidence interval; SBP, systolic blood pressure; CV, coefficient of variation.

results of ABPM and casual blood pressure measurement are summarized in Table 2. Table 1 shows that there were no significant differences between the two groups in baseline clinical characteristics, including the history of cerebrovascular disease and that of coronary artery disease. Table 2 shows that mean 24-h blood pressure, mean daytime blood pressure, mean nighttime blood pressure, casual blood pressure and pulse pressure were also similar between the two groups.

The median follow-up period was 34 months (range, 3–60 months). A total of 39 cardiovascular events occurred during the follow-up period. The events consisted of 3 cases of angina pectoris, 7 of acute myocardial infarction, 1 of coronary artery bypass graft surgery, 3 of sudden cardiac death, 3 of heart failure, 14 of cerebral infarction, 1 of cerebral hemorrhage, 5 of transient cerebral ischemic attack and 2 of aortic graft replacement surgery. Neither percutaneous coronary intervention nor acute aortic dissection was observed.

To investigate the impact of blood pressure variability on the onset of cardiovascular events, we plotted Kaplan-Meier curves for event-free survival and compared them between the two groups. Figure 1A shows that the rate of cardiovascular events was significantly higher in the high CV group than in the low CV group. When the patients were divided into two groups according to the mean value of SD of 24-h SBP, a significantly higher rate of cardiovascular events was observed in the high SD group (Fig. 1B). With respect to daytime SBP, patients with high CV values of daytime SBP as well as those with high SD values also had significantly more cardiovascular events (Fig. 1C, D). On the other hand, no difference in the rate of cardiovascular events was observed between the two groups when the mean value of CV or SD of nighttime SBP was used as a cut-off point (Fig. 1E, F).

To determine the independent predictive factors for cardiovascular events, the Cox's proportional hazards analysis was performed. This analysis identified male sex, high mean 24-h SBP and increased blood pressure variability (high CV value of 24-h SBP) as independent predictors for cardiovascular events (Table 3, A). In addition, the SD value of 24-h SBP was used as a variable rather than CV and the analysis was performed. History of coronary artery disease, high mean 24-h SBP and high SD value of 24-h SBP were significantly correlated with the onset of cardiovascular events (Table 3, B). Next, CV values of both daytime and nighttime blood pressure were used as variables. Male sex, history of coronary artery disease, high mean 24-h SBP and high CV value of daytime SBP were independent predictors (Table 3, C). Finally, the SD values of both daytime and nighttime blood pressure were used instead of the CV values and the analysis was performed. History of coronary artery disease, high mean 24-h SBP and high SD value of daytime SBP had significant correlations with the onset of cardiovascular events (Table 3, D).

Discussion

Hypertension is one of the leading causes of cardiovascular events (*1*) and the prevalence of hypertension increases with age (*2*). Therefore, it is important to clarify how to manage elderly hypertensive patients in clinical practice on the basis of their clinical features. Indeed, recent clinical trials have demonstrated that some antihypertensive drugs have a beneficial effect in elderly patients with isolated systolic hypertension (*13, 14*). However, the clinical significance of blood pressure variability remains to be determined in elderly hypertensive patients. Therefore, in this study, we analyzed the relationship between blood pressure variability and cardiovascular events in those patients.

Many studies concerning the clinical values of blood pressure variability have focused on circadian rhythm (*15–23*). Very recently, several clinical studies have been published to clarify the significance of blood pressure variability (*24–31*). The degree of blood pressure variability is related to hypertensive target organ damage (*24, 25*). The SD value of daytime blood pressure has a significant positive correlation with the progression of intima-media thickness of carotid arteries (*26*) and with the occurrence of lacunar infarction (*27*) in the hypertensive population. It has also been reported that the SD value of daytime blood pressure is correlated with left ventricular mass index both in hypertensive patients (*28*) and in the general population (*29*). In addition, an increase in the SD value of blood pressure variability is associated with cognitive impairment (*30*). Furthermore, it has been shown that a

high SD value of daytime blood pressure is an independent predictor for cardiovascular mortality in the general population (*31*). In addition to these studies, the present study on elderly patients with hypertension showed that high values of blood pressure variability of both 24-h blood pressure and daytime blood pressure were independent predictors of cardiovascular events in those specific patients.

The mechanisms underlying the positive correlation between blood pressure variability and the incidence of cardiovascular events could not be addressed in this study. The blood pressure variability is influenced by baroreflex regulation. The afferent fibers of this reflex arise from the aortic arch and carotid artery bifurcations and, therefore, in patients with arteriosclerosis, the afferent signal of the baroreflex may be decreased owing to low compliance of the arteriosclerotic vascular wall (*32*). In the present study, there was no significant difference in baseline clinical background or mean blood pressure values between the high CV group and low CV group. However, there is a possibility that subclinical arteriosclerosis may have been more advanced in the high CV group, and that blood pressure variability was increased as a consequence. This might explain the finding that more cardiovascular events occurred in the high CV group. On the other hand, another possibility is that blood pressure variability could have a direct effect on clinical outcome. The acute hemodynamic change observed in the high CV group might be a trigger for acute catastrophic events. In addition, blood pressure variability itself could induce vascular and organ damage, which might subsequently lead to cardiovascular events. Indeed, it has been reported that structural alteration of arteries (*33*) and cardiac hypertrophy (*34*) are observed in an animal model of high blood pressure variability.

Our study has some limitations. We used the discontinuous method of measuring blood pressure. This method is indeed less invasive to the patients but did not permit their full range of activity, and thus did not allow the recording of their full potential range of variability compared with the invasive continuous method. Indeed, we measured blood pressure only every 30 min. Because this measurement represents a low frequency sampling, the accuracy of blood pressure variability estimates assessed by ABPM may be reduced (*35*). In addition, our pilot study showed statistically significant correlations in terms of the short-term reproducibility of parameters obtained with 24-h ABPM, but absolute values of the correlation coefficient were not high enough. Furthermore, the possibility cannot be excluded that patients with excess nocturnal fall of blood pressure (extreme dippers), a condition that has already been shown to be associated with cerebrovascular disease (*17*), may have been defined as high CV patients in the present study. Moreover, it has been reported that some antihypertensive drugs reduce blood pressure variability (*36*). Because all patients were treated with one or two antihypertensive drugs in this study, there is a possibility that patients with lower blood pressure variability may have received more effective treatment, leading to better cardiovascular outcomes, despite the fact that the average blood pressure levels were identical between the two groups. Patients with and without organ damage at baseline were mixed together for analysis. It is possible that the significance of blood pressure variability in patients with organ damage could be different from that in patients without organ damage, because the autoregulatory function in response to acute change in blood pressure might be impaired in patients with organ damage, and thus these patients might be more susceptible to cardiovascular events. To clarify this point, subgroup analysis with a larger number of patients is required.

The present study was performed retrospectively in a longitudinal fashion. We made only a single measurement of 24-h blood pressure for the prediction of further events. Therefore, a prospective study with larger sample size and with repeated measurement should be conducted in the future to confirm the findings obtained in this study.

In conclusion, our data indicate that blood pressure variability is an independent risk factor for cardiovascular events in elderly hypertensive patients. This finding suggests that not only the average blood pressure level but also blood pressure variability should be taken into consideration for the management of elderly hypertensive patients.

References

1. Kannel WB: Blood pressure as a cardiovascular risk factor. Prevention and treatment. *JAMA* 1996; **275**: 1571–1576.
2. National High Blood Pressure Education Program Working Group: National High Blood Pressure Education Program Working Group report on hypertension in the elderly. *Hypertension* 1994; **23**: 275–285.
3. Vogat TM, Ireland CC, Black D, Camel G, Hughes G: Recruitment of elderly volunteers for a multicenter clinical trial: the SHEP Pilot Study. *Control Clin Trials* 1986; **7**: 118–133.
4. Floras JS, Hassan MO, Jones JV, Osikowska BA, Sever PS, Sleight P: Factors influencing blood pressure and heart rate variability in hypertensive humans. *Hypertension* 1988; **11**: 273–281.
5. Convanico V, De Caprio L, Vigorito C, *et al*: Differences in blood pressure profile between young and elderly hypertensive patients. *J Hum Hypertens* 1990; **4**: 405–409.
6. Rutan GH, Hermanson B, Bild DE, Kittner SJ, LaBaw F, Tell GS: Orthostatic hypotension in older adults. The Cardiovascular Health Study. *Hypertension* 1992; **19**: 508–519.
7. Appelegate WB, Davis BR, Black HR, Smith WM, Miller ST, Burlando AJ: Prevalence of postural hypotension at baseline in the Systolic Hypertension in the Elderly Program (SHEP) cohort. *J Am Geriatr Soc* 1991; **39**: 1057–1064.
8. Jansen RWMM, Lipsitz LA: Postprandial hypotension: epidemiology, pathophysiology and clinical management. *Ann Intern Med* 1995; **122**: 286–295.
9. Shimada K, Kitazumi T, Sadakane N, Ogura H, Ozawa T: Age-related changes in baroreflex function, plasma norepinephrine and blood pressure. *Hypertension* 1985; **7**: 113–117.
10. Gribbin B, Pickering LTG, Slight P, Petro R: Effects of age

and high blood pressure on baroreflex sensitivity in man. *Circ Res* 1971; **29**: 424.

11. Drayer JIM, Weber MA, DeYoung JL, Wyle FA: Circadian blood pressure patterns in ambulatory hypertensive patients. Effects of age. *Am J Med* 1982; **73**: 493–499.
12. White WB, Lund-Johansen P, McCabe EJ: Clinical evaluation of the Colin ABPM 630 at rest and during exercise: an ambulatory blood pressure monitoring with gas-powered cuff inflation. *J Hypertens* 1989; **7**: 477–483.
13. SHEP Cooperative Research Group: Prevention of stroke by antihypertensive drug treatment in older persons with isolated systolic hypertension. Final results of the Systolic Hypertension in the Elderly Program (SHEP). *JAMA* 1991; **265**: 3255–3264.
14. Staessen JA, Fagard R, Thijis L, *et al*: Randomized double-blind comparison of placebo and active treatment for older patients with isolated systolic hypertension. *Lancet* 1997; **350**: 757–764.
15. O'Brien E, Sheridan J, O'Malley K: Dippers and non-dippers. *Lancet* 1988; **ii**: 397.
16. Kario K, Shimada K: Change in diurnal blood pressure rhythm due to small lacunar infarct. *Lancet* 1994; **344**: 200.
17. Kario K, Matsuo T, Kobayashi H, Imiya M, Matsuo M, Shimada K: Nocturnal fall of blood pressure and silent cerebrovascular damage in elderly hypertensive patients. Advanced silent cerebrovascular damage in extreme dippers. *Hypertension* 1996; **27**: 130–135.
18. Shimada K, Kawamoto A, Matsubayashi K, Nishinaga M, Kimura S, Ozawa T: Diurnal blood pressure variations and silent cerebrovascular damage in elderly patients with hypertension. *J Hypertens* 1992; **10**: 875–878.
19. Suzuki Y, Kuwajima I, Kanemaru A, *et al*: The cardiac function reserve in elderly hypertensive patients with abnormal diurnal change in blood pressure. *J Hypertens* 1992; **10**: 173–179.
20. Verdecchia P, Shillaci G, Guerrieri M, *et al*: Circadian blood pressure changes and left ventricular hypertrophy in essential hypertension. *Circulation* 1990; **81**: 528–536.
21. Kikuya M, Sugimoto K, Katsuya T, *et al*: A/C1166 gene polymorphism of the angiotensin II type 1 receptor (AT1) and ambulatory blood pressure: the Ohasama Study. *Hypertens Res* 2003; **26**: 141–145.
22. Kario K, Schwartz JE, Gerin W, Robayo N, Maceo E, Pickering TG: Psychological and physical stress-induced cardiovascular reactivity and diurnal blood pressure variation in women with different work shifts. *Hypertens Res* 2002; **25**: 543–551.
23. Kario K, James GD, Marion R, Ahmed M, Pickering TG: The influence of work- and home-related stress on the levels and diurnal variation of ambulatory blood pressure and neurohumoral factors in employed women. *Hypertens Res* 2002; **25**: 499–506.
24. Parati G, Pomidossi G, Albini F, Malaspina D, Mancia G: Relationship of 24-hour blood pressure mean and variability to severity of target-organ damage in hypertension. *J Hypertens* 1987; **5**: 93–98.
25. Frattola A, Parati G, Cuspidi C, Albini F, Mancia G: Prognostic value of 24-hour blood pressure variability. *J Hypertens* 1993; **11**: 1133–1137.
26. Sander D, Kukla C, Klingelhofer J, Winbeck K, Conrad B: Relationship between circadian blood pressure patterns and progression of early carotid atherosclerosis: a 3-year follow-up study. *Circulation* 2000; **102**: 1536–1541.
27. Kukla C, Sander D, Schwarze J, Wittich I, Klingelhofer J: Changes of circadian blood pressure patterns are associated with the occurence of lucunar infarction. *Arch Neurol* 1998; **55**: 683–688.
28. Veerman DP, de Blok K, van Montfrans A: Relationship of steady state and ambulatory blood pressure variability to left ventricular mass and urinary albumin excretion in essential hypertension. *Am J Hypertens* 1996; **9**: 455–460.
29. Sega R, Corrao G, Bombelli M, *et al*: Blood pressure variability and organ damage in a general population: results from the PAMELA study (Pressioni Arteriose Monitorate E Loro Associazioni). *Hypertension* 2002; **39**: 710–714.
30. Kanemaru A, Kanemaru K, Kuwajima I: The effects of short-term blood pressure variability and nighttime blood pressure levels on cognitive function. *Hypertens Res* 2001; **24**: 19–24.
31. Kikuya M, Hozawa A, Ohokubo T, *et al*: Prognostic significance of blood pressure and heart rate variabilities: the Ohasama study. *Hypertension* 2000; **36**: 901–906.
32. Appenzeller O, Descarries L: Circulatory reflexes in patients with cerebrovascular disease. *N Engl J Med* 1964; **271**: 820–823.
33. Sasaki S, Yoneda Y, Fujita H, *et al*: Association of blood pressure variability with induction of atherosclerosis in cholesterol-fed rats. *Am J Hypertens* 1994; **7**: 453–459.
34. van Vliet BN, Hu L, Scott T, Chafe L, Montani JP: Cardiac hypertrophy and telemetered blood pressure 6 wk after baroreceptor denervation in normotensive rats. *Am J Physiol* 1996; **271**: R1759–R1769.
35. Parati G, Saul JP, Di Rienzo M, Mancia G: Spectral analysis of blood pressure and heart rate variability in evaluating cardiovascular regulation. A critical appraisal. *Hypertension* 1995; **25**: 1276–1286.
36. Mancia G, Omboni S, Rovogli A, Parati G, Zanchetti A: Ambulatory blood pressure monitoring in the evaluation of antihypertensive treatment: additional information from a large data base. *Blood Press* 1995; **4**: 148–156.

Renin-Angiotensin System Modulates Oxidative Stress–Induced Endothelial Cell Apoptosis in Rats

Masahiro Akishita, Kumiko Nagai, Hang Xi, Wei Yu, Noriko Sudoh, Tokumitsu Watanabe, Mica Ohara-Imaizumi, Shinya Nagamatsu, Koichi Kozaki, Masatsugu Horiuchi, Kenji Toba

Abstract—The role of the renin-angiotensin system in oxidative stress–induced apoptosis of endothelial cells (ECs) was investigated using a rat model and cultured ECs. EC apoptosis was induced by 5-minute intra-arterial treatment of a rat carotid artery with 0.01 mmol/L H_2O_2 and was evaluated at 24 hours by chromatin staining of *en face* specimens with Hoechst 33342. Although activity of angiotensin-converting enzyme in arterial homogenates was not increased, administration of an angiotensin-converting enzyme inhibitor temocapril for 3 days before H_2O_2 treatment inhibited EC apoptosis, followed by reduced neointimal formation 2 weeks later. Also, an angiotensin II type 1 (AT1) receptor blocker (olmesartan) inhibited EC apoptosis, whereas angiotensin II administration accelerated apoptosis independently of blood pressure. Next, cultured ECs derived from a bovine carotid artery were treated with H_2O_2 to induce apoptosis, as evaluated by DNA fragmentation. Combination of angiotensin II and H_2O_2 dose-dependently increased EC apoptosis and 8-isoprostane formation, a marker of oxidative stress. Conversely, temocapril and olmesartan reduced apoptosis and 8-isoprostane formation induced by H_2O_2, suggesting that endogenous angiotensin II interacts with H_2O_2 to elevate oxidative stress levels and EC apoptosis. Neither an AT2 receptor blocker, PD123319, affected H_2O_2-induced apoptosis, nor a NO synthase inhibitor, N^G-nitro-L-arginine methyl ester, influenced the effect of temocapril on apoptosis in cell culture experiments. These results suggest that AT1 receptor signaling augments EC apoptosis in the process of oxidative stress–induced vascular injury. (***Hypertension*. 2005;45:1188-1193.**)

Key Words: angiotensin ■ apoptosis ■ carotid arteries ■ endothelium ■ free radicals

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]

The renin-angiotensin system (RAS) is known to play a pivotal role in the process of vascular lesion formation such as atherosclerosis and restenosis after angioplasty. The expression of RAS components renin,[9] angiotensinogen,[10] angiotensin-converting enzyme (ACE),[11,12] and angiotensin II (Ang II) receptors[13] is upregulated in vascular lesions. Also, RAS inhibitors attenuate neointimal formation after vascular injury in animals[12,14] and endothelial dysfunction in humans.[15,16] The interaction between oxidative stress and the RAS, factors essential for the development of vascular disease, needs to be addressed. It has been demonstrated that RAS activation induces oxidative stress[17–20] and can enhance EC apoptosis in vitro.[20,21] However, it has not been elucidated whether the RAS plays a role in oxidative stress–induced vascular injury in vivo, particularly in EC apoptosis, an initial and important process in atherosclerosis.[1,22,23]

In this study, we first tested whether the RAS would augment EC apoptosis induced by brief exposure to H_2O_2 and the subsequent neointimal formation using a rat model.[24] Next, we used an in vitro model of H_2O_2-induced EC apoptosis to clarify the underlying cellular mechanism.

Methods

H_2O_2 Treatment of Carotid Artery

Ten- to 12-week-old male Wistar rats (Japan Clea; Tokyo, Japan) were used in this study. Maintenance of rats and surgical procedures for H_2O_2 treatment were performed as described previously.[24] Methods are detailed in the online data supplement (available online at http://www.hypertensionaha.org). All of the experimental protocols were approved by the animal research committee of the Kyorin University School of Medicine.

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From the Department of Geriatric Medicine (M.A., K.N., H.X., W.Y., N.S., K.K., K.T.), Kyorin University School of Medicine, Tokyo, Japan; Department of Geriatric Medicine (T.W.), Graduate School of Medicine, University of Tokyo, Japan; Department of Biochemistry (M.O.-I., S.N.), Kyorin University School of Medicine, Tokyo, Japan; Department of Medical Biochemistry (M.H.), Ehime University School of Medicine, Japan.

Correspondence to Masahiro Akishita, MD, PhD, Department of Geriatric Medicine, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. E-mail akishita-tky@umin.ac.jp

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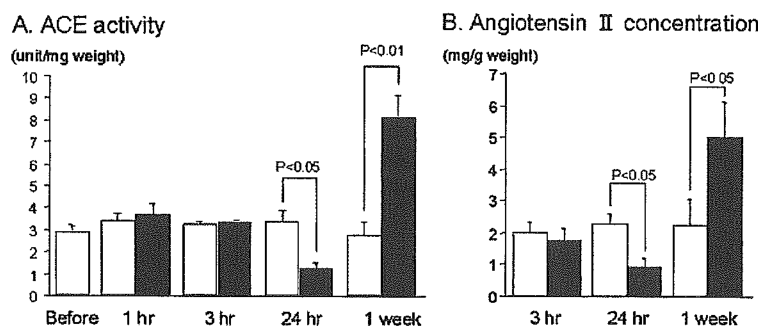

Figure 1. ACE activity and Ang II concentration in rat carotid artery after H_2O_2 treatment. Treated (closed bars) and contralateral (open bar) carotid arteries were harvested at the indicated time points after H_2O_2 treatment. ACE activity and Ang II concentration in tissue homogenates were measured using a pool of samples consisting of 6 to 10 arteries and were calibrated by the tissue wet weight. Values are expressed as mean±SEM of 5 to 6 independent pools.

Animal Groups and Blood Pressure Measurement

An ACE inhibitor, temocapril (10 mg/kg per day; donated by Sankyo Co, Ltd; Tokyo, Japan), or vehicle (40% ethanol) was administered orally using a feeding tube daily for 3 days. Separately, an Ang II type 1 (AT1) receptor blocker, olmesartan (1 mg/kg per day; donated by Sankyo Co, Ltd), or vehicle (40% ethanol) was administered orally for 3 days. Ang II was administered for 3 days using an osmotic minipump (Model 103D; Alza Corporation) prefilled with Ang II (0.7 mg/kg per day; Sigma), and implanted subcutaneously in the back. Hydralazine (25 mg/kg per day; Sigma) was orally administered alone for 5 days and subsequently with or without Ang II for 3 days before H_2O_2 treatment to abolish the effect of Ang II on blood pressure. On the last day of drug administration, blood pressure was measured with the animals in a conscious state by the tail-cuff method (BP-98A; Softron), and then H_2O_2 treatment was performed.

Measurement of ACE Activity and Ang II Concentration

At various time points after H_2O_2 treatment, the carotid arteries were dissected, weighed, and stored at −80°C. Pooled samples (n=6 to 10 for a pool) were homogenized with a polytron homogenizer in distilled water and centrifuged at 25 000*g* for 30 minutes at 4°C. ACE activity and Ang II concentration in the supernatants were measured using a colorimetric assay[12] and a sensitive radioimmunoassay, respectively. The values were calibrated by the tissue wet weight. ACE activity in the cell lysates of cultured ECs was measured using a colorimetric assay and calibrated by the protein concentration.

Evaluation of EC Apoptosis and Neointimal Formation in Carotid Artery

EC apoptosis was evaluated at 24 hours after H_2O_2 treatment as described previously.[24] Neointimal formation in the common carotid artery was evaluated 2 weeks after H_2O_2 treatment as described previously.[24] Methods are detailed in the online data supplement.

Induction of EC Apoptosis in Culture

ECs isolated from bovine carotid artery[25] were used at the fifth to seventh passage. When the cells had grown to 80% confluence, ECs were pretreated for 24 hours with culture medium containing the reagents that were tested in the experiments. Subsequently, after washing twice with Hank's balanced salt solution, the cells were exposed to H_2O_2 (0.01 to 0.2 mmol/L) diluted in Hank's balanced salt solution for 1.5 hours at 37°C to induce apoptosis. The cells were washed twice with Hank's balanced salt solution and then cultured in culture medium containing the reagents until assay.

The effects of temocapril, olmesartan, a NO synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME; Sigma), an Ang II type 2 (AT2) receptor blocker, PD123319 (Research Biochemical International), and Ang II (Sigma) were examined by adding them into the medium throughout the experiments.

Measurement of EC Apoptosis and Oxidative Stress Markers in Culture

For quantitative determination of apoptosis, we measured DNA fragmentation and caspase-3 activity at 24 hours after H_2O_2 treatment. DNA fragmentation was evaluated by histone-associated DNA fragments using a photometric enzyme immunoassay (EIA; Cell Death Detection ELISA; Roche) according to manufacturer instructions. Caspase-3 activity was measured using a colorimetric kit (Caspase-3 Colorimetric Activity Assay Kit; Chemicon) based on its activity to digest the substrate DVED according to manufacturer instructions.

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 manufacturer instructions. Intracellular oxidative stress levels were measured using 2′,7′-dichlorofluorescein (DCF) as described previously,[26] and the intensity values were calculated using the Metamorph software.

Real-Time Polymerase Chain Reaction

Real-time polymerase chain reaction (PCR) to quantify AT1 receptor mRNA in cultured ECs was performed using SYBR Green I (Sigma) and the ABI Prism 7000 Sequence Detection System (Applied Biosystems). Methods are detailed in the online data supplement.

Data Analysis

The values are expressed as mean±SEM in the text and figure data were analyzed using 1-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.

Results

ACE Activity in Carotid Artery After H_2O_2 Treatment

We examined whether H_2O_2 treatment would activate ACE and stimulate Ang II synthesis in the carotid artery. As shown in Figure 1A, ACE activity in tissue homogenates was not increased at 1 to 3 hours and, rather, was decreased at 24 hours, probably because of EC denudation.[24] Low ACE activity in the de-endothelialized artery is consistent with the previous finding[11,12] and was confirmed by measurement of ACE activity in the rat carotid artery, in which ECs were denuded ex vivo using a cotton swab (data not shown). In contrast, ACE activity was significantly increased at 1 week after H_2O_2 treatment, reflecting neointimal formation.[11,12,24] Ang II concentration in arterial homogenates showed similar changes to ACE activity after H_2O_2 treatment (Figure 1B).