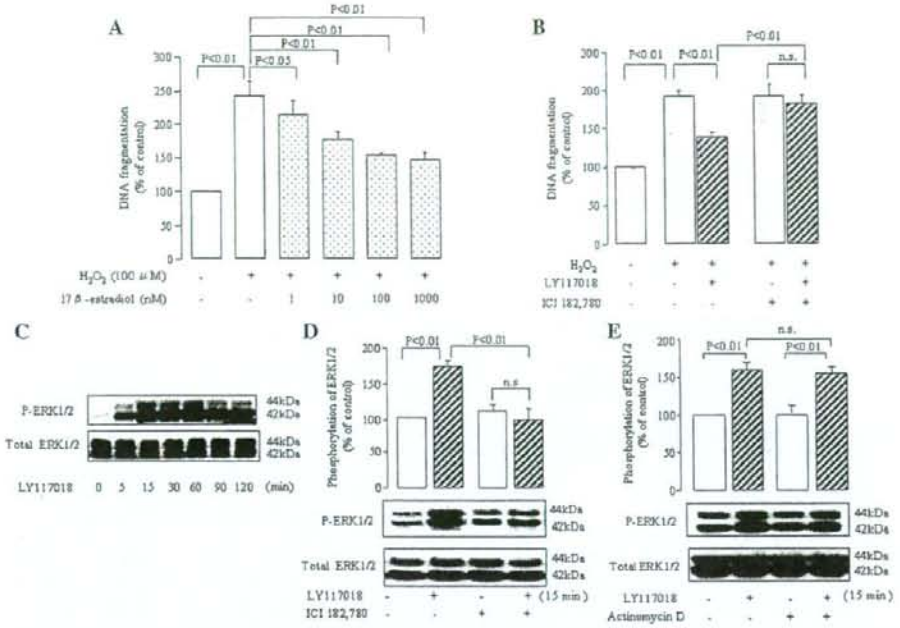

Fig. 4. Involvement of estrogen receptors in anti-apoptotic action of LY117018. At 70–80% confluence, BCEC were starved and exposed to H_2O_2 (100 μM) for 1 h as described in Materials and methods (A and B). (A) Various concentrations of 17β-estradiol (1 nM–1 μM) were added to the culture medium 30 min prior to exposure to H_2O_2 in the apoptosis assay. (B) In the estrogen receptor antagonist experiment, cells were pretreated with ICI 182,780 (10 μM) for 1 h before addition of 1 μM LY117018. Apoptosis was evaluated after 24 h of H_2O_2 treatment by means of DNA fragmentation (with a Cell Death Detection ELISA ^{plus} kit). Data are expressed as means ± S.E.M. Differences with a value of $P<0.05$ were considered statistically significant ($n=6$). (C, D and E) Serum-starved cells were stimulated with 1 μM LY117018 and harvested at the times indicated (C). In some groups, cells were pretreated with 10 μM ICI 182,780 (D) or 5 μg/ml actinomycin D (E) for 1 h before addition of LY117018 (1 μM, 30 min). Cell lysates were analyzed by Western blot as described in Materials and methods using a specific antibody against phospho-ERK1/2 (Thr202/Tyr204) or total ERK1/2. Representative blots and quantitative data evaluated by densitometry are shown ($n=3$). Values are expressed as means ± S.E.M. Differences with a value of $P<0.05$ were considered statistically significant.

was mediated through activation of another survival signal, the ERK1/2 pathway.

3.3. Involvement of estrogen receptors in anti-apoptotic action of LY117018

17β-Estradiol, an endogenous ligand for estrogen receptors, inhibited BCEC apoptosis in a concentration-dependent manner (Fig. 4A). 17β-Estradiol exerted an anti-apoptotic action at 1 nM, while LY117018 at 10 nM protected endothelial cells from apoptosis induced by H_2O_2 (Fig. 1). ICI 182,780, an estrogen receptor antagonist, significantly diminished the inhibitory effect of LY117018 on BCEC apoptosis (Fig. 4B). In addition, LY117018 per se rapidly increased the phosphorylation of ERK1/2 more than 5 min after its addition. Maximal phosphorylation was attained after 15 min of incubation (Fig. 4C). The LY117018-induced increase in ERK1/2 phosphorylation was significantly suppressed by ICI 182,780 (Fig. 4D). These results suggest that estrogen receptors are involved in the increased phosphorylation of ERK1/2 by LY117018.

To examine whether the LY117018-induced increase in ERK1/2 phosphorylation is due to a genomic or non-genomic action, the transcription inhibitor, actinomycin D, was added to BCEC prior to treatment with LY117018. The activation of ERK1/2 was not inhibited by actinomycin D (Fig. 4E). These results suggest that the anti-apoptotic activity of LY117018 is exerted through a non-genomic action.

4. Discussion

In the present study, we found that the raloxifene analogue, LY117018, inhibited BCEC apoptosis induced by H_2O_2. This inhibitory effect of LY117018 was concentration dependent. LY117018 at 10 nM protected endothelial cells from apoptosis by H_2O_2, while 17β-estradiol exerted an anti-apoptotic action at 1 nM. This may be explained by the difference in receptor ligand affinity between 17β-estradiol and LY117018. Indeed, the relative binding affinity of 17β-estradiol to estrogen receptor alpha is about 10 times higher than that of raloxifene in estrogen receptor-positive MCF-7 cells (Wijayaratne et al., 1999). The lower affinity of raloxifene for the estrogen receptor may be attributable to a structural difference. In addition, the concentrations of LY117018 used in our study might be relevant, because if we consider that the dose of raloxifene used in clinical settings is 120 mg/day, the serum concentration found in women treated with raloxifene is about 6 nM (Eli-Lilly, Indianapolis, IN, USA, unpublished data, 2003), which is close to the effective concentration of LY117018 in our experiments.

It has been reported that the inhibitory effect of raloxifene on bone absorption is mediated by direct binding with estrogen receptors. Endothelial cells express both estrogen receptor alpha (ER-alpha) and beta (ER-beta). In order to examine whether the anti-apoptotic effects of LY117018 are mediated by estrogen receptors, we examined the effects of a specific estrogen receptor antagonist, ICI 182,780. The anti-apoptotic effect of LY117018 was abolished by ICI 182,780. In addition, 17β-estradiol, an endogenous ligand for estrogen receptors, significantly inhibited apoptosis in BCEC. These observations suggest that LY117018 acts as an estrogen receptor agonist in endothelial cells, leading to endothelial cell survival. It has been reported that steroid hormones cause rapid responses, in minutes, through their membrane receptors. In recent years, several studies regarding the non-genomic actions of estradiol through estrogen receptors have been reported

(Razandi et al., 2003). In vascular cells, the roles of membrane estrogen receptors have been extensively investigated. Estrogen receptors mainly exist in the nucleus as ligand-dependent transcriptional factors, whereas a small amount of estrogen receptors in the cytoplasm do not enter the nucleus upon ligand stimulation and induce rapid signaling events (Pedram et al., 2002). LY117018 rapidly increased the phosphorylation of ERK1/2 after 5 min, and maximal phosphorylation was attained after 15 min of incubation. In addition, the increase in ERK1/2 phosphorylation was not inhibited by actinomycin D. These results suggest that the anti-apoptotic activity of LY117018 is exerted through a non-genomic action.

Recent studies support the idea that the induction of apoptosis by H_2O_2 is regulated by the balance between death signaling (p38, and JNK) and survival signaling (MEK/ERK1/2, and PI3-kinase/Akt) (Xia et al., 1995; Matsuzaki et al., 1999; Uchiyama et al., 2004). Indeed, in our study, H_2O_2 induced the phosphorylation of Akt, ERK1/2, JNK and p38. The p38 inhibitor, SB203580, and JNK inhibitor, SP600125, significantly decreased BCEC apoptosis induced by H_2O_2, whereas the PI3-kinase inhibitor, wortmannin, and MEK1 inhibitor, PD98059, significantly enhanced it. These results suggest that p38 and JNK act as cell death signals, whereas ERK1/2 and PI3-kinase/Akt act as survival signals in the process of BCEC apoptosis. Then we investigated the signaling pathways responsible for the anti-apoptotic effect of LY117018. Interestingly, LY117018 enhanced the phosphorylation level of ERK1/2 only, while it did not enhance the phosphorylation level of Akt or decrease that of p38 and JNK. In addition, PD98059 completely abolished the anti-apoptotic effect of LY117018, suggesting that the anti-apoptotic effect of LY117018 is mediated through enhancement of ERK1/2 signaling in vascular endothelial cells.

In conclusion, LY117018, an analogue of raloxifene, inhibits H_2O_2-induced endothelial apoptosis by activating ERK1/2, which is a non-genomic action via estrogen receptors. This study provides experimental evidence to support a novel therapeutic approach to pathological vascular conditions such as atherosclerosis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejphar.2008.04.052.

References

Akishita, M., Kozaki, K., Eto, M., Yoshizumi, M., Ishikawa, M., Toba, K., Orimo, H., Ouchi, Y., 1998. Estrogen attenuates endothelin-1 production by bovine endothelial cells via estrogen receptor. Biochem. Biophys. Res. Commun. 251, 17–21.

Barrett-Connor, E., Grady, D., Sashegyi, A., Anderson, P.W., Cox, D.A., Hoszowski, K., Rautaharju, P., Harper, K.D., MORE Investigators, 2002. Raloxifene and cardiovascular events in osteoporotic postmenopausal women: four-year results from the MORE (Multiple Outcomes of Raloxifene Evaluation) randomized trial. JAMA 287, 847–857.

Clarkson, T.B., 2007. Estrogen effects on arteries vary with stage of reproductive life and extent of subclinical atherosclerosis progression. Menopause 14, 373–384.

Cox, D.A., Sarkar, S., Harper, K., Barrett-Connor, E., 2004. Effect of raloxifene on the incidence of elevated low density lipoprotein (LDL) and achievement of LDL target goals in postmenopausal women. Curr. Med. Res. Opin. 20, 1049–1055.

Delmas, P.D., Bjarnason, N.H., Mitlak, B.H., Ravoux, A.C., Shah, A.S., Huster, W.J., Draper, M., Christiansen, C., 1997. Effects of raloxifene on bone mineral density, serum cholesterol concentrations, and uterine endometrium in postmenopausal women. N. Engl. J. Med. 337, 1641–1647.

Grady, D., Herrington, D., Bittner, V., Blumenthal, R., Davidson, M., Hlatky, M., Hsia, J., Hulley, S., Herd, A., Khan, S., Newby, L.K., Waters, D., Vittinghoff, E., Wenger, N., HERS Research Group, 2002. Cardiovascular disease outcomes during 6.8 years of hormone therapy: Heart and estrogen/progestin replacement study follow-up (HERS II). JAMA 288, 49–57.

Grady, D., Ettinger, B., Moscarelli, E., Plouffe Jr., L., Sarkar, S., Ciaccia, A., Cummings, S., 2004. Outcomes of raloxifene evaluation investigators. Safety and adverse effects associated with raloxifene: multiple outcomes of raloxifene evaluation. Obstet. Gynecol. 104, 837–844.

Hulley, S., Grady, D., Bush, T., Furberg, C., Herrington, D., Riggs, B., Vittinghoff, E., 1998. Randomized trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women. Heart and Estrogen/progestin Replacement Study (HERS) Research Group. JAMA 280, 605–613.

Johnell, O., Cauley, J.A., Kulkarni, P.M., Wong, M., Stock, J.L., 2004. Raloxifene reduces risk of vertebral fractures and breast cancer in postmenopausal women regardless of prior hormone therapy. J. Fam. Pract. 53, 789–796.

Kannel, W.B., Hjortland, M.C., McNamara, P.M., Gordon, T., 1976. Menopause and risk of cardiovascular disease: the Framingham study. Ann. Intern. Med. 85, 447–452.

Matsuzaki, H., Tamatani, M., Mitsuda, N., Namikawa, K., Kiyama, H., Miyake, S., Tohyama, M., 1999. Activation of Akt kinase inhibits apoptosis and changes in Bcl-2 and Bax expression induced by nitric oxide in primary hippocampal neurons. J. Neurochem. 73, 2037–2046.

Pedram, A., Razandi, M., Aitkenhead, M., Hughes, C.C., Levin, E.R., 2002. Integration of the non-genomic and genomic actions of estrogen. Membrane-initiated signaling by steroid to transcription and cell biology. J. Biol. Chem. 277, 50768–50775.

Razandi, M., Pedram, A., Park, S.T., Levin, E.R., 2003. Proximal events in signaling by plasma membrane estrogen receptors. J. Biol. Chem. 278, 2701–2712.

Ross, R., 1990. Mechanisms of atherosclerosis: a review. Adv. Nephrol. Necker Hosp. 19, 79–86.

Ross, R., 1999. Atherosclerosis — an inflammatory disease. N. Engl. J. Med. 340, 115–126.

Rossouw, J.E., Anderson, G.L., Prentice, R.L., LaCroix, A.Z., Kooperberg, C., Stefanick, M.L., Jackson, R.D., Beresford, S.A., Howard, B.V., Johnson, K.C., Kotchen, J.M., Ockene, J., Writing Group for the Women's Health Initiative Investigators, 2002. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. JAMA 288, 321–333.

Simoncini, T., De., Caterina, R., Genazzani, A.R., 1999. Selective estrogen receptor modulators: different actions on vascular cell adhesion molecule-1 (VCAM-1) expression in human endothelial cells. J. Clin. Endocrinol. Metab. 84, 815–818.

Simoncini, T., Varone, G., Fornari, L., Mannella, P., Luisi, M., Labrie, F., Genazzani, A.R., 2002. Genomic and nongenomic mechanisms of nitric oxide synthesis induction in human endothelial cells by a fourth-generation selective estrogen receptor modulator. Endocrinology 143, 2052–2061.

Sporn, M.B., Dowsett, S.A., Mershon, J., Bryant, H.U., 2004. Role of raloxifene in breast cancer prevention in postmenopausal women: clinical evidence and potential mechanisms of action. Clin. Ther. 26, 830–840.

Sudoh, N., Toba, K., Akishita, M., Ako, J., Hashimoto, M., Iijima, K., Kim, S., Liang, Y.Q., Ohike, Y., Watanabe, T., Yamazaki, I., Yoshizumi, M., Eto, M., Ouchi, Y., 2001. Estrogen prevents oxidative stress-induced endothelial cell apoptosis in rats. Circulation 103, 724–729.

Uchiyama, T., Engelman, R.M., Maulik, N., Das, D.K., 2004. Role of Akt signaling in mitochondrial survival pathway triggered by hypoxic preconditioning. Circulation 22, 3042–3049.

Wijayaratne, A.L., Nagel, S.C., Paige, L.A., Christensen, D.J., Norris, J.D., Fowlkes, D.M., McDonnell, D.P., 1999. Comparative analyses of mechanistic differences among antiestrogens. Endocrinology 140, 5828–5840.

Xia, Z., Dickens, M., Raingeaud, J., Davis, R.J., Greenberg, M.E., 1995. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 270, 1326–1331.

8. Ivry RB, Spencer RM, Zelaznik HN et al. The cerebellum and event timing. Ann N Y Acad Sci 2002;978:302–317.
9. Rondi-Reig L, Burguiere E. Is the cerebellum ready for navigation? Prog Brain Res 2005;148:199–212.
10. Schmahmann JD, Sherman JC. The cerebellar cognitive affective syndrome. Brain 1998;121(Pt 4):561–579.

NOCTURIA IN ELDERLY PEOPLE WITH HYPERTENSION—NO INFLUENCE OF LOW-DOSE THIAZIDE ADDED TO LOSARTAN

To the Editor: Most guidelines recommend thiazide-type diuretics as the preferred initial drugs for the treatment of hypertension. Thiazides, used in lower doses in elderly people with hypertension, are useful especially when used with an angiotensin II receptor blocker (ARB), which can antagonize the potassium-excreting effect of thiazides. It is also reported that drug-related adverse reactions are lower when thiazide-type diuretics are added to ARB monotherapy than with other combinations.[1,2] However, in these studies, consideration of the influence of thiazides on the geriatric syndrome is lacking. There could be a particular concern with regard to nocturia, a component of the geriatric syndrome that influences general health and quality of life (QOL) in elderly patients.[3] Some epidemiological studies have reported that diuretics are one of the risk factors for urinary problems in elderly people.[4] With this in mind, the effect of low-dose thiazide added to losartan monotherapy on nocturia in elderly hypertensives was examined.

Fifteen elderly patients with hypertension (mean age ± standard deviation = 76 ± 8, female/male = 6/9) who had been receiving losartan monotherapy (50 or 100 mg/d) for more than 1 month and had systolic blood pressure (BP) of 140 mmHg or greater or diastolic BP of 90 mmHg or greater were enrolled. They did not have any history of heart failure or other cardiovascular disease. Hydrochlorothiazide (HCTZ) at 12.5 mg/d was added to losartan and maintained for 3 months. Other medication was unchanged during the study; four patients were taking a statin, one was taking a sulfonylurea, but no patient was taking medication for gout or an overactive bladder. BP was measured at home and in the doctor's office early in the morning. Laboratory tests were performed in the morning after an overnight fast, and questionnaires on nocturia and health-related QOL were given to all patients.

As shown in Table 1, addition of low-dose HCTZ significantly reduced office BP after 1 month. The BP-lowering effect was sustained at 3 months and confirmed by morning home BP. No patient experienced worsening of nocturia during the study. On average, the frequency of nocturia was less at 1 month. At 3 months, the frequency of nocturia was similar to that at baseline. The result was unchanged if the subjects without nocturia were excluded from the analysis (1.3 ± 0.6 episodes/night at baseline to 1.2 ± 0.6 episodes/night after 3 months; n = 11, $P = .17$). In the QOL questionnaire (visual analog scale graded 0–5), sufficiency of sleep (3.4 ± 1.5 to 3.3 ± 1.6) and satisfaction with health (3.1 ± 1.0 to 3.2 ± 0.9) did not change significantly during the study period. Although serum creatinine and uric acid were significantly higher after 3 months, the changes were slight, and no subject showed an abnormal rise beyond the normal range. Fasting plasma glucose, serum total cholesterol, triglycerides, urea nitrogen, and potassium did not change significantly (Table 1 and data not shown).

Table 1. Changes in Blood Pressure (BP), Serum Parameters, and Nocturia After Addition of 12.5 mg/Day of Hydrochlorothiazide to Losartan

Parameter	Baseline	After 1 Month	After 3 Months
	Mean ± Standard Deviation		
Office systolic BP, mmHg	153 ± 17	140 ± 18†	137 ± 18†
Office diastolic BP, mmHg	87 ± 9	81 ± 10*	75 ± 10†
Home systolic BP, mmHg	144 ± 11	—	132 ± 11†
Home diastolic BP, mmHg	81 ± 9	—	77 ± 8
Glucose, mg/dL	107 ± 14	—	107 ± 13
Creatinine, mg/dL	0.82 ± 0.13	—	0.88 ± 0.15*
Uric acid, mg/dL	5.3 ± 1.2	—	6.1 ± 1.8*
Potassium, mEq/L	4.3 ± 0.3	—	4.2 ± 0.2
Nocturia, times per night	0.97 ± 0.81	0.67 ± 0.82*	0.87 ± 0.74

P< †.01, *.05 vs baseline according to paired t-test.
— = not determined.

This study is preliminary in terms of its size and noncontrolled design, but the results suggest that addition of low-dose HCTZ to losartan monotherapy effectively inhibits BP without worsening of nocturia in elderly people with hypertension. Because an ARB/thiazide combination is one of the most frequently prescribed regimens, the findings of the present study may provide useful information on urinary problems in elderly people with hypertension.

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REFERENCES

1. Oparil S, Barr E, Elkins M et al. Efficacy, tolerability, and effects on quality of life of losartan, alone or with hydrochlorothiazide, versus amlodipine, alone or with hydrochlorothiazide, in patients with essential hypertension. Clin Ther 1996;18:608–625.

2. Oparil S, Aurup P, Snavely D et al. Efficacy and safety of losartan/hydrochlorothiazide in patients with severe hypertension. Am J Cardiol 2001;87:721–726.
3. Asplund R. Nocturia in relation to sleep, health, and medical treatment in the elderly. BJU Int 2005;96(Suppl 1):15–21.
4. Johnson TM, Sattin RW, Parmelee P et al. Evaluating potentially modifiable risk factors for prevalent and incident nocturia in older adults. J Am Geriatr Soc 2005;53:1011–1016.

DISCLOSURE OF DEMENTIA DIAGNOSIS AND THE NEED FOR ADVANCE CARE PLANNING IN INDIVIDUALS WITH ALZHEIMER'S DISEASE

To the Editor: In a recent article in the *Journal of the American Geriatrics Society,* Carpenter and colleagues found that disclosure of a dementia diagnosis does not prompt a catastrophic emotional reaction in most people with dementia—not even in those who are cognitively only mildly impaired.[1] Companions of these patients also remained stable or even declined in their emotional stress immediately after the disclosure of dementia diagnosis. These findings suggest that physicians can provide a dementia diagnosis to a patient without fear of prompting strong emotional reactions.

In our experience, the disclosure of a diagnosis of dementia and adjustment to understanding all dimensions related to this information are a long-term process in which the patient's feelings develop and change over time. We examined the experiences of spousal caregivers of patients with Alzheimer's disease (AD) with many comorbidities and disabilities regarding the disclosure of dementia diagnosis and the subsequent need for advance care planning (ACP).

METHODS

The study included a survey of a random sample of 1,943 caregivers of persons with AD in Finland. All of the persons with AD had a confirmed diagnosis and received a compensation for AD medication from the state. Of the 1,434 respondents, 1,214 identified themselves as their spouse's caregiver. The mean age of the caregivers was 78.2, and that of the spouses with dementia was 80.5. Of the caregivers, 63% were female. The couples had long-lasting marriages (mean 52 years).[2] The mailed questionnaire included items on demographic characteristics and the physical and psychological symptoms and care needs of the persons with dementia. These questions and their validity have been described in detail elsewhere.[2]

The questions related to ACP had been used in previous interview studies in elderly people.[3,4] The ACP questions appear in Table 1.

RESULTS

Of the caregivers, 90% reported that dementia had been disclosed openly to their spouse; 97% also preferred that physicians openly inform the patients of the dementia diagnosis, although more than half of their spouses with AD had developed depressive symptoms after the disclosure. Of the caregivers, two-thirds felt that their awareness of their spouse's dementia caused them grief or symptoms of depression.[5]

After the disclosure of a diagnosis, a large proportion of caregivers felt a need for discussion about ACP with their physician. Of the caregivers, 59% expressed that they would like to discuss ACP with their spouse's physician, although only 6% reported that they had discussions related to ACP with a physician. Of the caregivers, fewer than one-third reported that they had discussed their spouse's medical care preferences with each other, and only 4% of the spouses with dementia had a written living will (LW). The couples in which the spouse with AD had a LW were generally better prepared for the loss of autonomy than the others.

DISCUSSION

This large epidemiological study of people with AD showed that physicians had openly disclosed their diagnosis to nearly all of the subjects, and depressive reactions over time were common in the patients with AD and their caregivers.

Table 1. Questions and Spousal Caregiver's Responses Relating to Advance Care Planning (ACP) of Home-Dwelling Persons with Alzheimer's Disease (AD) in Finland, 2005, According to Whether the Person with AD Had a Living Will (LW)

Question	Person with AD having a LW (n = 46)	Person with AD not having a LW (n = 1,127)	*P*-Value*
	Yes %		
Do you think that the follow-up care of your spouse's dementia is well arranged?	52.6	50.2	.77
Have you discussed your spouse's medical care preferences with each other?	63.2	26.1	<.001
Have you discussed these items with your spouse's physician?	31.0	5.1	<.001
Would you like to discuss ACP with your spouse's physician?	59.5	58.8	.93
Have you made a financial arrangement with your bank or some broader authorization to make your spouse's financial arrangements easier?	61.4	36.8	<.001
Have you requested for a legal guardian for your spouse to make financial or other arrangements easier?	13.6	3.8	<.001

*Differences in proportions between two groups were tested using the chi-square test.

Cilostazol Inhibits Oxidative Stress–Induced Premature Senescence Via Upregulation of Sirt1 in Human Endothelial Cells

Hidetaka Ota, Masato Eto, Mitsunobu R. Kano, Sumito Ogawa, Katsuya Iijima, Masahiro Akishita, Yasuyoshi Ouchi

Objective—Cilostazol, a selective inhibitor of PDE3, has a protective effect on endothelium after ischemic vascular damage, through production of nitric oxide (NO). The purpose of the present study was to clarify the molecular mechanisms underlying the preventive effect of treatment with cilostazol on oxidative stress–induced premature senescence in human endothelial cells.

Methods and Results—Prematurely senescent human umbilical vein endothelial cells (HUVECs) were induced by treatment with hydrogen peroxide (H_2O_2) as judged by senescence-associated β-galactosidase assay (SA-βgal), cell morphological appearance, and plasminogen activator inhibitor-1 (PAI-1) expression. Treatment with H_2O_2 caused 93% of the cells to be SA-βgal positive, whereas 46% of cilostazol (100 μmol/L)-treated cells were positive. HUVECs treated with other cAMP-elevating agents and DETA-NO showed a reduction of SA-βgal–positive cells as well. Cilostazol increased phosphorylation of Akt at Ser^{473} and of endothelial nitric oxide synthase (eNOS) at Ser^{1177}, with a dose-dependent increase in Sirt1 expression. Moreover, the effect of cilostazol on premature senescence was abrogated through inhibition of Sirt1.

Conclusions—Our results indicated that cilostazol exerted protective effects against endothelial senescence and dysfunction, and enhancement of NO production is a key mediator in upregulation of Sirt1. (***Arterioscler Thromb Vasc Biol.* 2008;28:1634-1639**)

Key Words: cilostazol ■ eNOS ■ Sirt1 ■ endothelial senescence

The phenomenon of human aging is known to be a critical cardiovascular risk factor. Cellular senescence of endothelial cells has been proposed to be involved in endothelial dysfunction and atherogenesis.[1] The lesions of human atherosclerosis have been extensively studied histologically, and these studies have demonstrated that there are vascular cells that exhibit the morphological features of cellular senescence.[2]

See accompanying article on page 1577

The telomere hypothesis is a widely accepted explanation of the occurrence of cellular senescence.[3] Cessation of cell division after extended propagation in culture for a few weeks or months is related to the attrition of telomeres, which is termed replicative senescence. In addition to telomere attrition, some stressors such as oxidative stress elicit similar growth arrest within just a few days, referred to as stress-induced premature senescence (SIPS). Both types of senescence are accompanied by a specific set of changes in cell function, morphology, and gene expression.[4] In addition to the above changes, recognized biomarkers of senescent cells include staining for β-galactosidase at pH of 6.0 as opposed to endogenous lysosomal enzyme detected at pH of 4.0 in normal cells.[5]

According to the free-radical theory, reactive oxygen species (ROS) may be potential candidates responsible for senescence and age-related diseases, and on production of high levels of ROS, the redox balance is disturbed and cells shift into a state of oxidative stress, which subsequently leads to premature senescence with shortening telomeres.[6]

A PDE3 inhibitor, cilostazol, is used as a vasodilating antiplatelet drug for treating intermittent claudication, and in preclinical studies was shown to have a protective effect on endothelial cells by increasing eNOS activity.[7] Cilostazol increases intracellular cAMP content accordingly and activates protein kinase A (PKA) or PI3K/Akt signaling.[8] As recently shown, endothelial NO can protect against a state of oxidative stress, and activation of eNOS and subsequent production of NO delay endothelial cellular senescence.[9,10]

In yeast, Sir2 (silent information regulator-2) has been identified as an NAD^+-dependent histone deacetylase.[11]

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Mammalian sirtuin 1 (Sirt1), the closest homolog of Sir2, regulates the cell cycle, senescence, apoptosis, and metabolism, by interacting with a number of molecules, including p53, PML, and PPAR-γ.[12–14] A recent study showed that production of NO by caloric restriction increases Sirt1 expression and suggested that eNOS may be involved in regulating the expression of Sirt1 in murine white adipocytes.[15] Therefore, we consider that the protective effect of cilostazol against vascular senescence may be attributed to upregulation of Sirt1.

In the present study, cilostazol inhibited oxidative stress–induced premature senescence, and the increased expression of Sirt1 by this drug played a critical role in prevention of endothelial senescence.

Materials and Methods

Cilostazol was kindly provided by Otsuka Pharmaceutical Co Ltd, Tokyo, Japan. Forskolin, rolipram, N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME) and LY294002 were purchased from Sigma. Myristoylated cell-permeable PKA inhibitor peptide sequence[14–22] amide (PKAI) was from Alexis Biochemicals. (Z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino] diazen-1-IM1,2 diolate (DETA-NO), S-nitrosoacetyl penicillamine (SNAP), 8 Br-cGMP, and 8 Br-cAMP were from Cayman Chemical. *N*-acetyl-cystein (NAC) was purchased from Calbiochemo.

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were purchased from CAMBREX (Walkersville, Md), and maintained in endothelial growth medium (EGM-2, EGM-2 singleQuots, CAMBREX). Population doubling levels (PDL) were calculated as described previously,[16] and all experiments were performed at PDL of 8 to 9.

Measurement of cAMP Level

HUVECs were plated in 96-well plates at a density of 5×10^3 cells per well and cultured overnight. After 15-minute incubation with cilostazol, the medium was aspirated and a lysis buffer was added. cAMP concentration was determined using a cAMP EIA kit (Amersham Biosciences) according to the manufacturer's instructions.

Inhibition of Sirt1

Proliferating cells were washed 3 times with growth medium and exposed for 24 hours to the indicated concentrations of sirtinol (Calbiochem) or nicotinamide (NAM, Wako Chemical Industries) diluted in medium. After exposure, the dishes were washed 3 times with inhibitor-free medium and cultured. Proliferating cells were transfected with 200 pmol/L siRNA for Sirt1 (GAT GAA GTT GAC CTC CTC A[14] and TGA AGT GCC trichloroacetic acid (TCA) GAT ATT A) or control siRNA (Darmacom Co.) using silMPORTER (Upstate Cell Signaling Solutions).

Senescence-Associated β-Galactosidase (SA-βgal) Staining

HUVECs were grown in 100-mm collagen-coated dishes to 80% confluence. HUVECs were pretreated with vehicle (0.05% DMSO), cilostazol (1 to 100 μmol/L), forskolin (0.1 to 1 μmol/L), rolipram (10 to 100 μmol/L), DETA-NO (50, 100 μmol/L), or NAC (3, 5 mmol/L) diluted in EGM-2 medium for 3 days. HUVECs were washed 3 times with EGM-2 and then treated for 1 hour with 100 μmol/L H_2O_2 diluted in EGM-2. After the treatment, HUVECs were trypsinized, reseeding at the density of 1×10^5 in 60-mm dishes and cultured with EGM-2 containing these compounds for 10 days. At 10 days after treatment with H_2O_2, HUVECs were fixed and the proportion of SA-βgal–positive cells was determined as described by Dimri et al.[5]

NOS Activation Assay

NOS activity was determined using an NOS assay kit (Calbiochem) according to the manufacturer's instructions.

BrdU Incorporation Assay

BrdU incorporation was analyzed using a commercial kit (Roche).

Immunoblotting

Cells were lysed on ice for 1 hour in buffer (50 mmol/L Tris-HCl, pH 7.6, 150 mmol/L NaCl, 1%NP-40, 0.1%SDS, 1 mmol/L dithiothreitol, 1 mmol/L sodium vanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 10 mmol/L sodium fluoride). Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After blocking, the filters were incubated with the following antibodies; antiphospho-eNOS (Ser1177), antiphospho-Akt (Ser473), anti-Akt (Cell Signaling Technology), antieNOS (BD Transduction Laboratories), antiacetyl-p53 (Lys373/382), antip53, anti-Sirt1 (Santa Cruz Biotechnology Inc), anti-PAI-1 (Molecular Innovations Inc), and anti-β-actin (Sigma). After washing and incubation with horseradish peroxidase-conjugated antirabbit or antimouse IgG (Amersham) for 1 hour, the antigen-antibody complexes were visualized using an enhanced chemiluminescence system (Amersham).

Real-Time Quantitative Reverse Transcription

Expression of Sirt1 in HUVECs was measured by quantitative RT-polymerase chain reaction (PCR). Total RNA in HUVECs was isolated with ISOGEN (Nippon gene Inc). After treatment with Rnase-free Dnase for 30 minutes, total RNA (50 ng/μL) was reverse transcribed with random hexamers and oligo d(T) primers. The expression level of Sirt1 relative to GAPDH was determined by means of staining with SYBR green dye and a LineGene fluorescent quantitative detection system (Bioflux Co), as recommended by the manufacturer. Primer quality was verified by dissociation curve analysis, the slopes of standard curves, and reactions without RT. The following primers were used: Sirt1 (forward (F) 5′-CCTGACTTCAGATCAAGAGACGGT-3′; reverse (R) 5′-CTGATTAAAAATGTCTCCACGAACAG-3′, GAPDH F 5′-ACCAC-AGTCCATGCCATCAC-3′; R 5′-TCCACCACCCTGTTGCTGTA-3′).

Animal Experiments

The animal experiments were approved by our institutional review board. Ten-week-old SPF male wild-type BALB/c mice (n=40, weighing approximately 25 g) were supplied by Charles River Laboratories Inc. Animals were housed under a 12-hour light/dark cycle and fed a normal diet. These mice were administered 25 mg/kg paraquat (1,1-dimethyl-4,4-bipyridinium) (Wako Chemical) by intraperitoneal injection. Then mice were randomly assigned to 2 treatment groups (control group, n=20; cilostazol group, n=20). The each group received gavage administration of vehicle alone or cilostazol 60 mg/kg/d for their lifetime. We made diabetic mice (n=40) by a single intraperitoneal injection of streptozotocin (STZ; 60 mg/kg, Sigma). Tail blood glucose was assayed 3 days after injection using glucose test strips (Roche). The mice were killed by cervical dislocation. The aorta was removed after systemic perfusion with phosphate-buffered saline (PBS) for histological examination. The proportion of SA-βgal–positive cells was analyzed by NIH image software. The primary antibody was purified rat antimouse CD31 (platelet endothelial cell adhesion molecule; PECAM-1) monoclonal antibody from pharmingen. ROS were measured with 2′, 7′-Dichlorodihydrofluorescein, diacetate (DCF) (Sigma). As previously described by Shi et al,[17] the aorta was rapidly removed and placed in oxygenated (12% O_2, 5% CO_2) physiological salt solution (PSS) of the following composition (NaCl 130 mmol/L, KCl 4.7 mmol/L, $CaCl_2$ 1.6 mmol/L, $MgSO_4$ 1.17 mmol/L, $NaHCO_3$ 14.9 mmol/L, KH_2PO_4 1.18 mmol/L, EDTA 0.026 mmol/L, glucose 1.0 mmol/L). The living aorta was carefully isolated, cannulated (24G, Terumo Co Ltd) at both ends, pressured, and loaded with DCF solution (10 μmol/L) for 10 minutes. Then the aorta was washed by PSS 3 times, embedded in OCT medium, and cryosectioned.

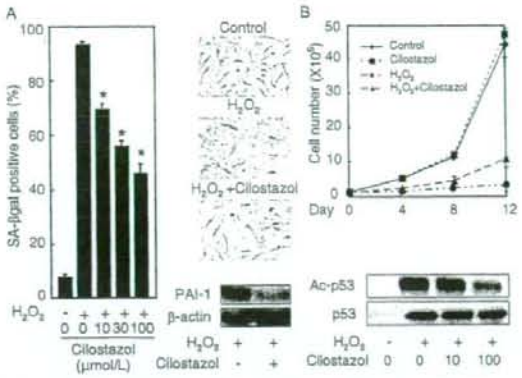

Figure 1. A, Cilostazol inhibited H_2O_2 (100 μmol/L)-induced premature senescent phenotype in HUVECs as judged by SA-βgal staining (*$P<0.05$, n=3), morphological changes, and PAI-1. B, Cell growth curve, acetylation of p53 (Ac-p53) at lysine 373/382, and total protein of p53 were evaluated at 10 days after addition of H_2O_2.

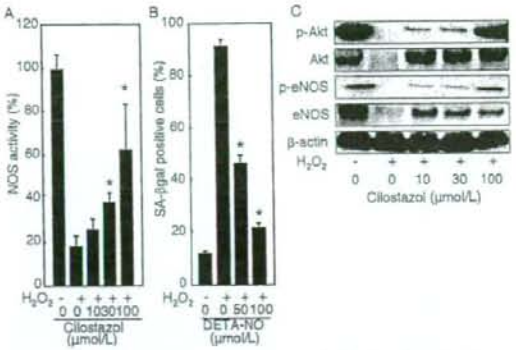

Figure 2. A, NOS activity was measured after treatment with cilostazol. B, DETA-NO inhibited H_2O_2 (100 μmol/L)-induced premature senescent phenotype in HUVECs as judged by SA-βgal staining. (*$P<0.05$, n=3). C, Expression of phospho-eNOS (Ser1177), phospho-Akt (Ser473), Akt, and eNOS in cilostazol-treated cells.

TOTO-3 for nuclear staining, secondary antibodies (Alexa Fluor 488 donkey antirat IgG and Alexa Fluor 594 donkey antirat IgG), and antifade reagent were from Molecular Probe (Invitrogen). Fluorescent images were taken and analyzed using a confocal laser microscope (LSM510, Carl Zeiss MicroImaging Co Ltd). Urinary 8-Hydroxydeoxyguanosine (8-OhdG) and creatinine were measured using a DNA damage ELISA kit (Stressgen) and creatinine assay kit (Cayman chemical), respectively.

Telomerase Assay

Telomerase activity was measured with 2 μg protein using a telomerase PCR-ELISA kit according to the manufactures instructions (Chemicon, Temecula).

Data Analysis

Values are shown as mean±SEM in the text and figures. Differences between the groups were analyzed using 1-way analysis of variance, followed by Bonferroni test. Probability values less than 0.05 were considered significant.

Results

Cilostazol Inhibits Oxidative Stress–Induced Premature Senescence in Human Endothelial Cells

To investigate the effect of cilostazol on the senescent phenotype in HUVECs, we induced premature endothelial senescence by addition of H_2O_2 100 μmol/L for 1 hour. We found that treatment with cilostazol inhibited the senescent phenotype as judged by SA-βgal assay and enlarged and flattened cell morphological appearance at 10 days. Under treatment with H_2O_2, 93% of cells were SA-βgal positive, versus only 46% of cilostazol (100 μmol/L)-treated cells under the same oxidative conditions (Figure 1A). We found that HUVECs treated with other cAMP-elevating agents showed a reduction of SA-βgal–positive cells as well (forskolin 1 μmol/L; 51%, rolipram 100 μmol/L; 53%). Treatment with cilostazol decreased the specific senescent morphological changes (Figure 1A). Expression of PAI-1 was decreased by treatment with cilostazol (Figure 1A). Treatment with cilostazol restored the rate of BrdU incorporation in prematurely senescent HUVECs (supplemental Figure I, available online at http://atvb.ahajournals.org). In parallel with this, telomerase activity was increased by treatment with cilostazol (supplemental Figure I). Moreover, we examined the effect on cell growth for 12 days after treatment with vehicle, H_2O_2 and cilostazol. Addition of H_2O_2 decreased cell number of HUVECs and treatment with cilostazol recovered it (Figure 1B). p53 plays a pivotal role in cellular senescence. Therefore, we examined the expression and acetylation of p53 at Lys373/382, one of the critical targets of Sirt1. As shown in Figure 1B, we observed that H_2O_2 increased the expression and acetylation of p53, and treatment with cilostazol decreased the acetylation of p53.

Enhancement of cAMP Production and eNOS Activity Induced by Cilostazol

When HUVECs were treated with cilostazol, the cAMP level significantly increased in a concentration-dependent manner at cilostazol concentrations of 1 and 100 μmol/L (data not shown). In the presence of H_2O_2, cilostazol increased eNOS activity (Figure 2A), expression of eNOS, and the phosphorylation of eNOS at Ser^{1177} in parallel with the phosphrylation of Akt at Ser^{473} (Figure 2C). Although exposure to H_2O_2 affected the total amount of eNOS and Akt, treatment with cilostazol reverted their expression to nearly normal levels (Figure 2C). To investigate the effect of NO on the senescent phenotype in HUVECs, we treated these cells with an NO donor, DETA-NO (100 μmol/L). DETA-NO–treated HUVECs showed decreased SA-βgal–positive cells (Figure 2B), and an increased rate of BrdU incorporation and telomerase activity (supplemental Figure I). These results suggest that the protective effect against a senescent phenotype may be attributed to an increased of NO via eNOS activation by cilostazol.

Treatment With Cilostazol Increased Sirt1 Expression

To explore the mechanism by which cilostazol prevents from premature endothelial senescence, we considered that an increase in NO production could promote the longevity gene,

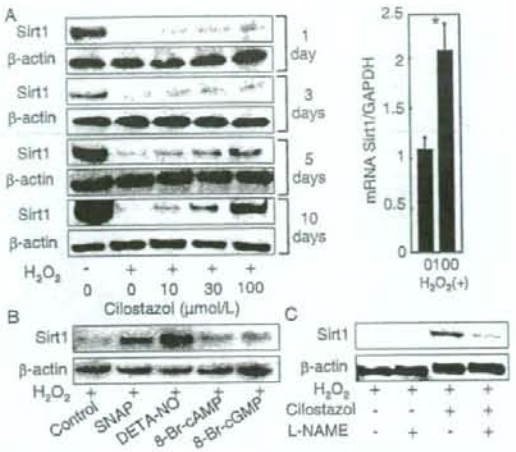

Figure 3. A, Sirt1 protein and mRNA in the presence of H_2O_2 (100 μmol/L). Sirt1 expression was increased by treatment with SNAP (100 μmol/L), DETA-NO (50 μmol/L), 8 Br-cGMP (1 mmol/L), or 8 Br-cAMP (1 mmol/L) (B) and decreased by treatment with L-NAME (20 μmol/L) for 6 hours (C). *$P<0.05$.

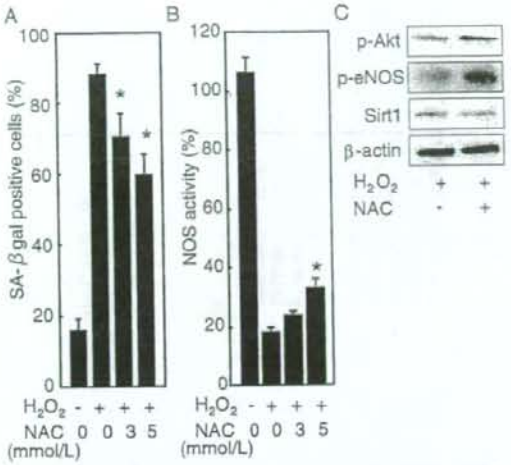

Figure 4. A, Treatment with NAC (0, 3, 5 mmol/L) inhibited H_2O_2 (100 μmol/L)-induced premature senescent phenotype in HUVECs as judged by SA-βgal staining (n=3). B, NOS activity after treatment with NAC. C, Expression of phospho-eNOS (Ser1177), phospho-Akt (Ser473), and Sirt1 in NAC (5 mmol/L)-treated cells. *$P<0.05$.

Sirt1. We found that cilostazol significantly increased Sirt1 mRNA and protein in a concentration-dependent manner for 10 days after treatment with H_2O_2 (Figure 3A). In contrast, Sirt1 mRNA and protein were not altered in the absence of H_2O_2 treatment (data not shown). To determine whether the expression of Sirt1 was regulated by the increase in NO production, we exposed prematurely senescent HUVECs to either an NO donor (such as DETA-NO or SNAP), a cAMP analog (8 Br-cAMP), or a cGMP analog (8 Br-cGMP). After these treatments, the expression of Sirt1 protein was markedly higher than that in untreated cells (Figure 3B). Furthermore, treatment with an NOS inhibitor, L-NAME, decreased Sirt1 expression (Figure 3C). To clarify the molecular mechanisms by which cilostazol induces SIRT1 expression, we examined the effect of protein kinase inhibitors on the cilostazol-induced phosphrylation of eNOS, Akt and expression of Sirt1 (supplemental Figure II). In the absence of H_2O_2 treatment, PKAI and LY294002 inhibited the cilostazol-induced phosphorylation of eNOS at Ser^{1177}. The cilostazol-induced phosphorylation of Akt at Ser^{473} was inhibited by LY294002, however the inhibition by PKAI was not significant. Sirt1 expression was not altered by treatment with PKAI or LY294002. In the presence of H_2O_2 treatment, PKAI and LY294002 showed the similar effect on the cilostazol-induced phosphorylation of eNOS at Ser^{1177} and Akt at Ser^{473}, but Sirt1 expression was significantly decreased.

Cilostazol Dose Not Have a Function of Direct Scavenger of Hydrogen Peroxide

It is possible that cilostazol may function as an antioxidant drug. Therefore, we examined the effect of NAC, another antioxidant, on Sirt1 expression, phosphorylation of Akt, and NOS activity as well as senescence markers. As shown in Figure 4A, treatment with NAC (0, 3, 5mmol/L) significantly decreased SA-βgal activity. Phosphorylation of Akt and NOS activity was increased by treatment with NAC (Figure 4B and 4C). However, Sirt1 expression was not altered (Figure 4C). To clarify the effect of cilostazol or NAC on H_2O_2, we examined whether cilostazol or NAC could scavenge H_2O_2 radicals. We performed a cell-free, horseradish peroxidase–coupled oxidation analysis.[18] We observed that NAC scavenged H_2O_2 significantly, but cilostazol not (supplemental Figure III). These results indicate that inhibiting H_2O_2-induced senescence by cilostazol may not be attributable to its direct antioxidative effect such as NAC.

Inhibition of Sirt1 Abrogates the Protective Effect of Cilostazol Against Premature Senescence

To determine the role of endogenous Sirt1 in premature senescence, HUVECs were treated with a Sirt1 chemical inhibitor, sirtinol, a physiological Sirt1 inhibitor, NAM or Sirt1 siRNA. Knockdown of Sirt1 with siRNA was confirmed by Western blotting. As shown in Figure 5A and 5B, Sirt1 inhibition abrogates the effect of cilostazol on SA-βgal activity and the senescent specific morphological changes. Likewise, we found that Sirt1 inhibition had a similar effect to DETA-NO treatment (data not shown). Increased phosphorylation of eNOS at Ser^{1177} and decreased expression of PAI-1 by cilostazol were no longer observed when Sirt1 was inhibited (Figure 5C). These results indicate that Sirt1 could play an important role in the protective effect of cilostazol against a senescent phenotype.

Administration of Cilostazol Inhibits Vascular Endothelial Senescence Induced by Oxidative Stress in BALB/c Mice

To investigate whether cilostazol has a protective effect on vascular endothelial senescence induced by oxidative stress in vivo, we administrated paraquat, a herbicide that generates

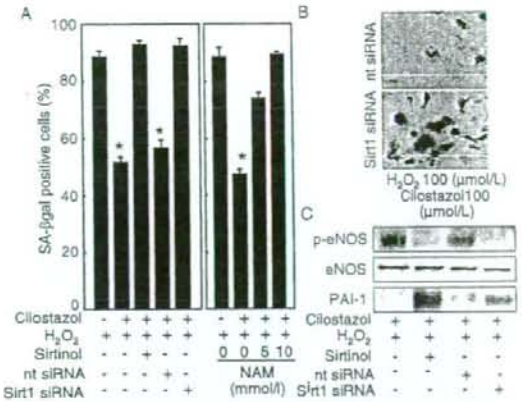

Figure 5. Inhibition of Sirt1 abrogates effect of cilostazol (100 μmol/L) against a premature senescence phenotype as shown by SA-βgal staining (n=3) (A), morphological changes (B), and expression of phospho-eNOS (Ser1177) and PAI-1 (C). Sirt1 was inhibited by sirtinol (100 μmol/L), NAM (5, 10 mmol/L), or Sirt1 siRNA. nt indicates nontargeted. *$P<0.05$.

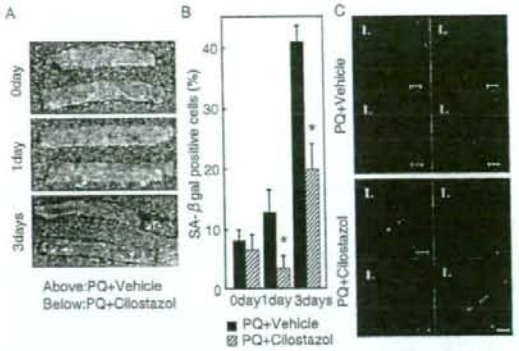

Figure 6. A, SA-βgal staining of thoracic arteries from BALB/c mice with cilostazol at 0, 1, and 3 days after treatment with paraquat (PQ). B, The number of SA-βgal staining cells in cilostazol-treated thoracic arteries. C, Immunofluorescent staining for Sirt1 (green), PECAM-1 (red), and TOTO-3 (blue). Representative samples (n=40) shown. *$P<0.05$.

superoxide, to BALB/c mice. We performed resection of the thoracic artery of these mice and compared the senescent phenotype in the presence and absence of cilostazol. The number of SA-βgal–staining cells was significantly increased in untreated thoracic arteries, but was decreased in cilostazol-treated thoracic arteries (Figure 6A and 6B). Cross-sections of arteries stained with SA-βgal showed that positive cells were mostly located on the luminal surface and stained for CD-31, indicating that blue staining originated from vascular endothelial cells and not from the extracellular matrix (supplemental Figure IV). To estimate the degree of DNA damage caused by paraquat, we measured urinary 8-OhdG, a marker of DNA damage from oxidative stress. Urinary 8-OHdG level was decreased after cilostazol treatment (supplemental Figure IV). Immunostaining of the sections for Sirt1 showed that Sirt1 expression was increased in aortic endothelial cells by treatment with cilostazol (Figure 6C). To estimate the antioxidative effect of cilostazol on vasculature, we used DCF, cell-permeable fluorogenic probe, to measure ROS within cells by detection of enzymatically formed H_2O_2. The intensity of green fluorescence indicating DCF-positive cells was markedly increased in untreated thoracic arteries, which was decreased in cilostazol-treated thoracic arteries (supplemental Figure IV). The number of DCF-positive endothelial cells was decreased in cilostazol-treated thoracic arteries (supplemental Figure IV). Next, we used STZ diabetic mice in which the endothelial senescence documented.[19] The treatment with cilostazol decreased SA-βgal–positive endothelial cells (supplemental Figure V).

Discussion

As previously reported,[20] the concentration of cilostazol (60 mg/kg/d) we administrated in this study was within clinical relevance. In vitro experiments, we used cilostazol at 0 to 100 μmol/L and confirmed a concentration-dependent trend. Given the average plasma concentration of cilostazol orally administered to humans (100 mg/body/d) is about 2 to 10 μmol/L and may be partially higher in our body, our used concentration of cilostazol is comparable to clinical.

Previous studies have shown that overexpression of Sirt1 antagonizes cellular senescence through acetylation of p53 with localization of the PML body.[13] Recently, we reported that Sirt1 overexpression prevented the development of oxidative stress–induced premature senescence in human endothelial cells.[21] Senescence of endothelial cells leads to endothelial dysfunction and may result in advanced atherosclerotic lesions.[2] In fact, it has been reported that endothelial cells in samples of human aortia with atherosclerosis exhibited a senescence-like phenotype, increased expression of PAI-1,[22] and decreased production of NO.[1] NO production and eNOS expression are severely limited in senescent endothelial cells.[23] Although NO is known to be involved in reducing oxidative stress and the progression of atherosclerosis, the present study suggested that the NO-mediated prevention of premature senescence was attributable to Sirt1 function. These findings implicate the NO-Sirt1 axis as one of the fundamental determinants of endothelial senescence, and the role of Sirt1 as a driver of cellular stress resistance and longevity is noteworthy in the context of its expression profile.

The free-radical theory of aging proposes that degenerative senescence is largely the result of the cumulative effect of ROS. In this study, we used paraquat mice as an oxidative stress model. Moreover, we studied the effect of cilostazol on endothelial senescence used by STZ-diabetic mice as more suitable for clinical settings. In addition, Takase et al recently reported that cilostazol had exhibited an antiatherosclerotic effect on vasculature in ApoE-deficient mice.[24] Therefore, we suggest that cilostazol has a beneficial effect on vasculature in clinical settings.

Cilostazol-induced NO production by eNOS activation via a cAMP/PKA- and PI3K/Akt-dependent mechanism was previously confirmed in the porcine thoracic aorta by an ESR[25] technique and in clinical practice by endothelial-dependent vasodilation.[26] Our results showed that cilostazol phosphorylated Akt via a PKA-independent mechanism (sup-

plemental Figure II). It is suggested that both cAMP/PKA and PI3K/Akt signaling pathways are involved in cilostazol-induced phosphorylation of eNOS, however the contribution of these signaling to the upregulation of Sirt1 in the presence of H_2O_2 is more critical than that of in the absence of H_2O_2. Therefore, we suggest that upregulation of Sirt1 by cilostazol is modulated via cAMP/PKA, PI3K/Akt, and eNOS-dependent mechanism under oxidative conditions, but further investigation is needed to elucidate why there is discrepancy of Sirt1 expression.

A recent study showed DETA-NO, an NO-donor, and eNOS transfection activated hTERT and delayed endothelial senescence, indicating that eNOS has an antiatherosclerotic effect even in cases of advanced atherosclerosis. It is therefore suggested that incrased NO bioavailability by other pharmaceutical products such as 3-hydroxy-3-methylglutaryl (HMG)-coenzyme A (CoA) reductase inhibitors or agents with phytoestrogenic properties such as resveratrol may exert a protective effect against endothelial senescence, and this possibility deserves further investigation.

In summary, we showed that cilostazol inhibited oxidative stress–induced premature senescence, and subsequently enhancement of Sirt1 expression played a critical role in inhibition of a senescent phenotype in human endothelial cells. Our results suggest that NO production by cilostazol has a protective effect against endothelial senescence and dysfunction.

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Disclosures

None.

References

1. Minamino T, Miyauci H, Yoshida T, Ishida Y, Yoshida H, Komuro I. Endothelial cell senescence in human atherosclerosis: role of telomere in endothelial dysfunction. *Circulation*. 2002;105:1541–1544.
2. Burrig KF. The endothelium of advanced arteriosclerotic plaques in humans. *Arterioscler Thromb*. 1991;11:1678–1689.
3. Liu JP. Studies of the molecular mechanisms in the regulation of telomerase activity. *FASEB J*. 1999;13:2091–2104.
4. Yang J, Chang E, Cherry AM, Bangs CD, Oei Y, Bodnar A, Bronstein A, Chiu CP, Herron GS. Human endothelial cell life extension by telomerase expression. *J Biol Chem*. 1999;274:26141–26148.
5. Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linskens M, Rubelj I, Pereira-Smith O, et al. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc Natl Acad Sci USA*. 1995;92:9363–9367.
6. Kurz DJ, Decary S, Hong Y, Trivier E, Akhmedov A, Erusalimsky JD. Chronic oxidative stress compromises telomere integrity and accelerates the onset of senescence in human endothelial cells. *J Cell Sci*. 2004;117:2417–2426.
7. Kambayashi J, Liu Y, Sun B, Shakur Y, Yoshitake M, Czerwiec F. Cilostazol as a unique antithrombotic agent. *Curr Pharm Des*. 2003;9:2289–2302.
8. Hashimoto A, Miyakoda G, Hirose Y, Mori T. Activation of endothelial nitric oxide synthase by cilostazol via a cAMP/protein kinase A- and phosphatidylinositol 3-kinase/Akt-dependent mechanism. *Atherosclerosis*. 2006;189:350–357.
9. Hayashi T, Matsui-Hirai H, Miyazaki-Akita A, Fukatsu A, Funami J, Ding QF, Kamalanathan S, Hattori Y, Ignarro LJ, Iguchi A. Endothelial cellular senescence is inhibited by nitric oxide: implications in atherosclerosis associated with menopause and diabetes. *Proc Natl Acad Sci USA*. 2006;103:17018–17023.
10. Vasa M, Breitschopf K, Zeiher AM, Dimmeler S. Nitric oxide activates telomerase and delays endothelial cell senescence. *Circ Res*. 2000;87:540–542.
11. Braunstein M, Rose AB, Holmes SG, Allis CD, Broach JR. Transcriptional silencing in yeast is associated with reduced nucleosome acetylation. *Genes Dev*. 1993;7:592–604.
12. Vaziri H, Dessain SK, Ng Eaton E, Imai SI, Frye RA, Pandita TK, Guarente L, Weinberg RA. hSIR2 (SIRT1) functions as an NAD-dependent p53 deacetylase. *Cell*. 2001;107:149–159.
13. Langley E, Pearson M, Faretta M, Bauer UM, Frye RA, Minucci S, Pelicci PG, Kouzarides T. Human SIR2 deacetylates p53 and antagonizes PML/p53-induced cellular senescence. *EMBO J*. 2002;21:2383–2396.
14. Picard F, Kurtev M, Chung N, Topark-Ngarm A, Senawong T, Machado De Oliveira R, Leid M, McBurney MW, Guarente L. Sirt1 promotes fat mobilization in white adipocytes by repressing PPAR-gamma. *Nature*. 2004;429:771–776.
15. Nisoli E, Tonello C, Cardile A, Cozzi V, Bracale R, Tedesco L, Falcone S, Valerio A, Cantoni O, Clementi E, Moncada S, Carruba MO. Calorie restriction promotes mitochondrial biogenesis by inducing the expression of eNOS. *Science*. 2005;310:314–317.
16. Maciag T, Hoover GA, Stemerman MB, Weinstein R. Serial propagation of human endothelial cells in vitro. *J Cell Biol*. 1981;91:420–426.
17. Shi Y, Man RY, Vanhoutte PM. Two isoforms of cyclooxygenase contribute to augmented endothelium-dependent contractions in femoral arteries of 1-year-old rats. *Acta Pharmacol Sin*. 2008;29:185–192.
18. Frew JE, Jones P, Scoles G. Spectrophotometric determination of hydrogen peroxide and organic hydroperoxides at low concentrations in aqueous solution. *Analytica chemica acta*. 1983;155:139–150.
19. Yokoi T, Fukuo K, Yasuda O, Hotta M, Miyazaki J, Takemura Y, Kawamoto H, IchijoH, Ogihara T. Apoptosis signal-regulating kinase 1 mediates cellular senescence induced by high glucose in endothelial cells. *Diabetes*. 2006;55:1660–1665.
20. Akiyama H, Kudo S, Shimizu T. The absorption, distribution and excretion of a new antithrombotic and vasodilating agent, cilostazol, in rat, rabbit, dog and man. *Arzneimittelforschung*. 1985;35:1124–1132.
21. Ota H, Akishita M, Eto M, Iijima K, Kaneki M, Ouchi Y. Sirt1 modulates premature senescence-like phenotype in human endothelial cells. *J Mol Cell Cardiol*. 2007;43:571–579.
22. Albrecht EW, Stegeman CA, Heeringa P, Henning RH, van Goor H. Protective role of endothelial nitric oxide synthase. *J Pathol*. 2003;199:8–17.
23. Sato I, Morita I, Kaji K, Ikeda M, Nagao M, Murota S. Reduction of nitric oxide producing activity associated with in vitro aging in cultured human umbilical vein endothelial cell. *Biochem Biophys Res Commun*. 1993;195:1070–1076.
24. Takase H, Hashimoto A, Okutsu R, Hirose Y, Ito H, Imaizumi T, Miyakoda G, Mori T. Anti-atherosclerotic effect of cilostazol in apolipoprotein-E knockout mice. *Arzneimittelforschung*. 2007;57:185–191.
25. Nakamura T, Houchi H, Minami A, Sakamoto S, Tsuchiya K, Niwa Y, Minakuchi K, Nakaya Y. Endothelium-dependent relaxation by cilostazol, a phosphodiesteras III inhibitor, on rat thoracic aorta. *Life Sci*. 2001;69:1709–1715.
26. Watanabe K, Ikeda S, Komatsu J, Inaba S, Suzuki J, Sueda S, Funada J, Kitakaze M, Sekiya M. Effect of cilostazol on vasomotor reactivity in patients with vasospastic angina pectoris. *Am J Cardiol*. 2003;92:21–25.

Geriatr Gerontol Int 2008; 8: 93–100

ORIGINAL ARTICLE: EPIDEMIOLOGY, CLINICAL PRACTICE AND HEALTH

White matter lesions as a feature of cognitive impairment, low vitality and other symptoms of geriatric syndrome in the elderly

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Aim: White matter lesions (WML) are common findings on magnetic resonance imaging (MRI) in elderly persons. In this study, we analyzed the relation of WML with global cognitive function, depression, vitality/volition, and 19 symptoms of geriatric syndrome in Japanese elderly patients who attended three university geriatric outpatient clinics.

Methods: Two hundred and eighty-six subjects (103 men and 183 women; mean ± standard deviation age, 74.5 ± 7.8 years) were included in this study. MRI scans were performed for the diagnosis of WML, and the severity of periventricular and deep white matter hyperintensities (PVH and DWMH) was rated semiquantitatively. Concurrently, all subjects underwent tests of cognitive function, depressive state and vitality, and were examined for 19 symptoms of geriatric syndrome.

Results: The study subjects showed cognitive decline, depression and low vitality, all to a mild extent. Univariate linear regression analysis showed a negative correlation between the severity of WML and cognitive function or vitality. Multiple logistic analysis revealed that the severity of WML was a significant determinant of cognitive impairment and low vitality, after adjustment for confounding factors such as age, sex and concomitant diseases. PVH and/or DWMH score was significantly greater in subjects who exhibited 13 out of 19 symptoms of geriatric syndrome. Logistic regression analysis indicated that WML were associated with psychological disorders, gait disturbance, urinary problems and parkinsonism.

Conclusion: WML were associated with various symptoms of functional decline in older persons. Evaluating WML in relation to functional decline would be important for preventing disability in elderly people.

Keywords: deep white matter hyperintensity, geriatric syndrome, periventricular hyperintensity, white matter lesion.

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Introduction

Brain magnetic resonance imaging (MRI) has markedly enhanced the chance of detecting characteristic hyperintense signals in the periventricular and subcortical areas on T2-weighted images, even in asymptomatic older persons.[1] These lesions are known as white matter lesions (WML), leukoaraiosis or white matter (periventricular and subcortical) hyperintensities.[2–4] WML, which accompany symptoms of gait abnormalities,[5–7] urinary symptoms[5,8,9] and cognitive impairment,[4,10,11] are reported to be associated with aging,[12–14] hypertension,[14] diabetes[15] and atherosclerosis.[5] There is poor understanding of the pathogenesis of the lesions, and it remains unknown whether WML are mere innocuous radiological changes that appear as a result of the aging process,[2,3,10] or whether they are one of the causal factors of the functional decline in elderly people.

Geriatric syndrome is a group of symptoms that are related to daily life, and the comorbidity triggers the loss of independence of elderly persons. Hence, evaluation of geriatric syndrome is important for the physical and mental care of the elderly. To address the pathological significance of WML in the global cognitive and psychological functions, and in geriatric syndrome in representative Japanese elderly subjects, we organized a group of geriatric outpatient clinics, and investigated the clinical manifestations of WML in those patients. Especially, we analyzed the relation of WML with global cognitive function, depressive state, vitality/volition and 19 symptoms of geriatric syndrome.

Methods

Subjects

This was a multicenter study performed at three different university geriatric outpatient clinics in Japan under the organization of a Longevity Science Research Grant from the Ministry of Health, Labor and Welfare of Japan (H15-Choju-013). Two hundred and eighty-six consecutive subjects (103 men and 183 women; mean ± standard deviation [SD] age, 74.5 ± 7.8 years) were included in this study: 187 at Kyorin University Hospital, 74 at Chiba University Hospital, and 25 at Nagoya University Hospital, from January 2004 to January 2005.

The diagnosis of dementia was made according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV). The definition of hypertension was systolic blood pressure (BP) of more than 140 mmHg or diastolic BP of more than 90 mmHg, or receiving antihypertensive drugs. The definition of diabetes was glycosylated hemoglobin A1c of more than 6.5%, or receiving antidiabetic drugs. The definition of hyperlipidemia was total cholesterol of more than 5.72 mmol/L, triglyceride of more than 1.70 mmol/L, or receiving antihyperlipidemic drugs.

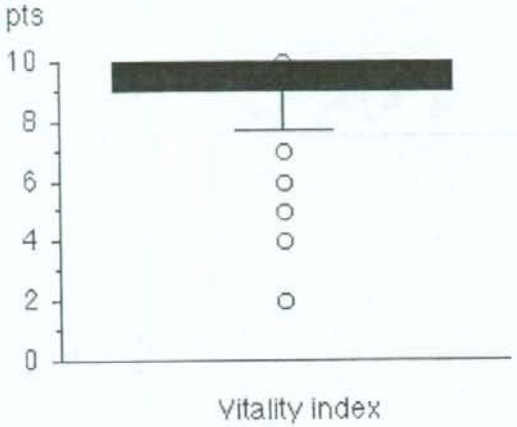

Figure 1 Distribution of vitality index. All subjects underwent assessment of vitality index as a measure of vitality related to activities of daily living (waking pattern, communication, feeding, getting on and off the toilet, rehabilitation and other activities; 2 points each; range, 0–10).

All subjects underwent the following assessment of global cognitive and psychological function. Cognitive function was evaluated by Mini-Mental State Examination (MMSE).[16] In this examination, we focused on calculation (serial subtraction of 7 from 100) to evaluate attention and working memory (part of the frontal lobe function). We also performed verbal fluency or word recollection test by asking the subjects to name as many vegetables as possible, which is also indicative of the frontal lobe function. Depression was evaluated by the 15-item Geriatric Depression Scale (GDS-15), which consists of 15 dichotomous questions for screening depressive symptoms in elderly subjects (range, 0–15).[17] Vitality index was used to measure vitality or volition in daily life (waking pattern, communication, feeding, getting on and off the toilet, rehabilitation and other activities; 2 points each; range, 0–10).[18] A full score can be maintained until one is severely disabled in cognition or function. The distribution of vitality index in the subjects of this study is shown in Figure 1.

We examined symptoms of geriatric syndrome: 19 dichotomous questions about hallucinations, delusions, insomnia, vertigo, paralysis, numbness, gait disturbance, tripping, falls, pollakiuria, urinary incontinence, constipation, decreased appetite, weight loss, apathy, speech impairment, swallowing difficulty, tremor and muscle stiffness.

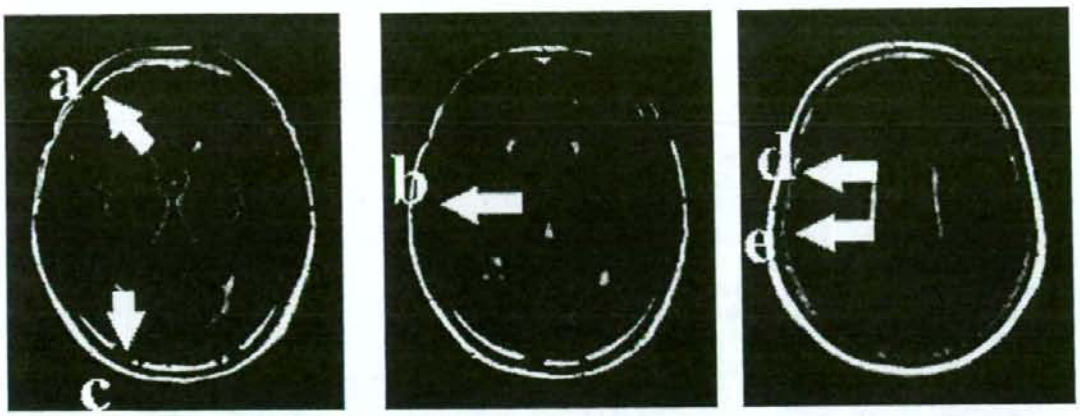

Figure 2 Evaluation of periventricular hyperintensity (PVH). PVH were evaluated in six regions in three slices: (a) adjacent to the frontal horns, (b) lateral ventricular body, (c) occipital horns, (d) frontal central semiovale in the parietal region and (e) occipital centrum semiovale in the parietal region in both hemispheres. Each area was rated as five grades according to the method of Junque *et al.*: (0) no hyperintensities; (1) <25% of the brain area; (2) 25–50%; (3) 50–75%; and (4) >75%.[11] The sum of all grades in the six regions was defined as the PVH score (range, 0–24).

Magnetic resonance imaging

Magnetic resonance imaging scans were performed for the diagnosis of WML and cerebral infarction on 1.5-T scanners (Toshiba, Nasu, Japan). T1-weighted images (repetition time [TR], 496 ms; echo time [TE], 12 ms), T2-weighted images (TR, 4280 ms; TE, 105 ms), and fluid-attenuated inversion-recovery (FLAIR)-weighted images (TR, 8000 ms; TE, 105 ms; 5-mm slice thickness) were obtained in the axial plane. MRI images were examined to differentiate between WML, characterized by isointense signals on T1-weighted images and hyperintense signals on T2-weighted and FLAIR images, and cerebral infarction, characterized by hypointense signals on T1-weighted images and hyperintense signals on T2-weighted and FLAIR images.

White matter lesions were classified as periventricular hyperintensities (PVH), which adjoined the lateral ventricle, and deep white matter hyperintensities (DWMH), located in the deep white matter apart from the lateral ventricles.

Periventricular and deep white matter hyperintensity scores

Periventricular hyperintensities were evaluated in six regions in three slices: adjacent to the frontal horns, lateral ventricular body, occipital horns, frontal central semiovale in the parietal region, and occipital centrum semiovale in the parietal region in both hemispheres (Fig. 2). Each area was rated as five grades according to the systematic quantification method developed by Junque *et al.*: (0) no hyperintensities; (1) less than 25% of the brain area; (2) 25–50%; (3) 50–75%; and (4) more than 75%.[11] The sum of all grades in the six regions was defined as the PVH score (range, 0–24).

Deep white matter hyperintensities were evaluated in the frontal, temporal, parietal and occipital lobes, and in the basal ganglia in both hemispheres (Fig. 3). Each lesion was rated as three grades according to the diameter by the study of de Groot *et al.*: (1) 1–3 mm; (2) 3–10 mm; and (3) more than 10 mm. The sum of all grades in five regions in both hemispheres was defined as the DWMH score.[4] Analysis was performed assuming that the white matter scores of PVH and DWMH were quantitative interval scales.

Statistical analysis

The relationship between two continuous variables such as MMSE, GDS-15 or vitality index, and WML (PVH or DWMH) score was analyzed by univariate linear regression analysis, and the correlation was analyzed by means of Pearson's simple correlation coefficients. Statistical significance was set at $P < 0.05$.

The relation of cognitive impairment or low vitality with PVH score or DWMH score was assessed by means of multivariate logistic regression analysis with adjustment for age, sex, hypertension, diabetes, hyperlipidemia and past history of cerebrovascular disease, of which all variables other than age were treated as categorical data. Cognitive impairment and low vitality were defined as an MMSE score of 23 or less[19] and a vitality index of 9 or less, respectively. Odds ratios and 95% confidence interval were calculated from the, coefficients and their standard errors.

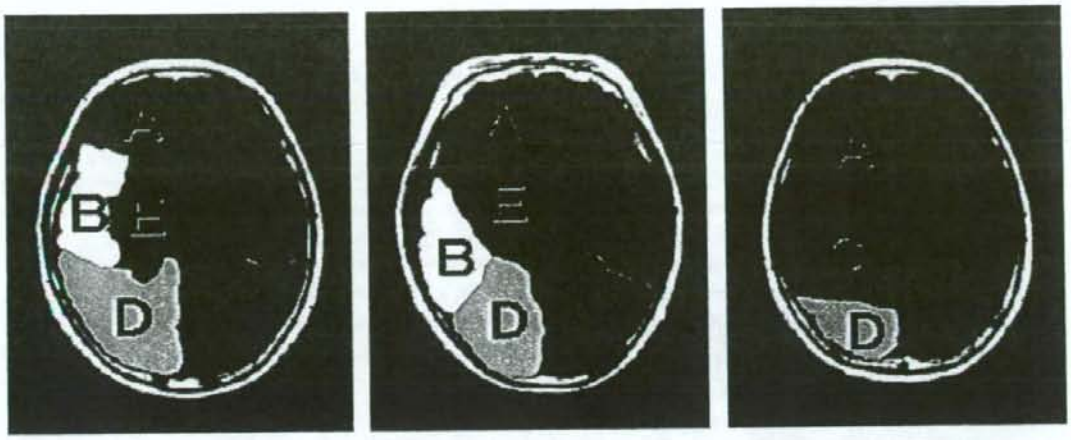

Figure 3 Evaluation of deep white matter hyperintensities (DWMH). DWMH were evaluated in the (A) frontal, (B) temporal, (C) parietal and (D) occipital lobes, and (E) in the basal ganglia in both hemispheres. Each lesion was rated as three grades according to diameter by the method of de Groot *et al.*: (1) 1–3 mm; (2) 3–10 mm; and (3) >10 mm.[4] The sum of all grades in five regions in both hemispheres was defined as the DWMH score.

Periventricular hyperintensity score or DWMH score was compared between subjects who did or did not exhibit each symptom of geriatric syndrome and analyzed by Student's *t*-test. When the difference was considered to be significant ($P < 0.05$), the difference was further assessed by means of multivariate logistic regression analysis with adjustment for age, sex, hypertension, diabetes, hyperlipidemia and past history of cerebrovascular disease.

Ethical considerations

This study was approved by the ethical committees of the institutes involved in this project. We explained this study clearly, and obtained written consent from all participants and their guardians (mainly family members). All the data were stored and analyzed carefully to preserve the subjects' anonymity and protect their privacy.

Results

Clinical data

The clinical characteristics of the study subjects are shown in Table 1. The mean age of subjects was 74.5 ± 7.8 years (mean ± SD), and subjects aged 65 or older comprised 88.1%. The mean body mass index was 21.8 ± 3.3 kg/m^2 and none of the subjects were obese. Of the subjects, 10.1% had experienced stroke or other cerebrovascular disease and 22.7% were smokers. Hypertension, diabetes and hyperlipidemia were present in 50.7%, 27.3% and 50.0% of the subjects, respectively.

White matter lesions

Periventricular hyperintensities and DWMH were observed in 77.7% and 96.7% of the total subjects, respectively. The mean score of PVH and DWMH was 5.5 ± 4.8 and 35.5 ± 39.8, respectively (Table 1). Pearson's correlation analysis showed a strong positive correlation between PVH score and DWHM score ($r = 0.56$, $P < 0.0001$). In relation to aging, a positive correlation was found between PVH score and age ($r = 0.34$, $P < 0.0001$), and between DWMH score and age ($r = 0.28$, $P < 0.0001$).

Cognitive and psychological assessment

The mean score of MMSE, GDS-15 and vitality index was 23.1 ± 5.3, 5.0 ± 3.5 and 9.4 ± 1.2 points, respectively, indicating that the subjects showed cognitive decline, depression and decreased vitality, all to a mild extent. Given that a score of 23 or below on MMSE is regarded as the presence of cognitive impairment,[19] 47.5% of the subjects fell into this category. The causes of cognitive impairment were Alzheimer disease (AD; 53.3%), vascular dementia (VaD; 16.4%), combined dementia of AD and VaD (9.0%) and other types of dementia (21.3%). Pearson's correlation analysis revealed a negative correlation between PVH score and MMSE, PVH score and vitality index, DWMH score and MMSE, and DWMH score and vitality index,

Table 1 Clinical characteristics of study subjects

	Prevalence ($n = 286$)	Mean ± standard deviation
Clinical characteristics		
Age (years)		74.5 ± 7.8
Women (%)	74.0	
Height (m)		1.55 ± 0.08
Bodyweight (kg)		52.4 ± 10.6
Body mass index (kg/m^2)		21.8 ± 3.3
Systolic blood pressure (mmHg)		135.3 ± 20.2
Diastolic blood pressure (mmHg)		76.3 ± 11.8
Prevalence of complications		
Hypertension (%)	50.7	
Diabetes (%)	27.3	
Hyperlipidemia (%)	50.0	
Past history of cerebrovascular disease (%)	10.1	
Smoking (%)	22.7	
Cognitive and psychological assessment		
Mini-Mental State Examination (0–30 points)		23.1 ± 5.3
Geriatric depression scale (0–15 points)		5.0 ± 3.5
Vitality index (0–10 points)		9.4 ± 1.2
White matter lesions		
Periventricular hyperintensities (points)	5.5 ± 4.8	
Deep white matter hyperintensities (points)	35.5 ± 39.8	

Table 2 Relationship between white matter lesions and global cognition (MMSE), depressive state (GDS-15) and vitality (vitality index)

	Linear regression	
	PVH score	DWMH score
MMSE	−0.380**	−0.272**
GDS-15	0.022	−0.066
Vitality index	−0.432**	−0.184*

Univariate linear regression analysis: *$P < 0.01$, **$P < 0.0001$. DVMH, deep white matter hyperintensity; GDS-15, 15-item Geriatric Depression Scale; MMSE, Mini-Mental State Examination; PVH, periventricular hyperintensity.

respectively (Table 2). It was also found that calculation (serial subtraction of 7 from 100) was negatively correlated with PVH score ($r = -0.156$, $P = 0.04$, data not shown), and verbal fluency (naming as many vegetables as possible) was negatively correlated with PVH score ($r = -0.216$, $P < 0.01$, data not shown). On the other hand, no significant correlation was found between PVH score and GDS-15, or between DWMH score and GDS-15. Multiple logistic analysis revealed that PVH score and DWMH score remained significant determinants of cognitive impairment (MMSE, ≤23) and low vitality (vitality index, ≤9) after adjustment for age, sex, presence of hypertension, diabetes, hyperlipidemia and past history of cerebrovascular disease (Table 3).

One hundred and ninety subjects reported symptoms of geriatric syndrome. The frequency is shown in Table 4. Frequent symptoms (>20%) were tripping (32.1%), constipation (26.3%), gait disturbance (23.2%) and pollakiuria (22.1%). Student's *t*-test showed that PVH score was significantly greater in subjects who exhibited the following symptoms of geriatric syndrome: hallucinations, delusions, gait disturbance, tripping, falls, pollakiuria, urinary incontinence, weight loss, apathy, swallowing difficulty, tremor and muscle stiffness. Multiple logistic analysis revealed that PVH score remained a significant determinant of hallucinations, tripping, pollakiuria, urinary incontinence, weight loss, apathy and swallowing difficulty after adjustment for age, sex, presence of hypertension, diabetes, hyperlipidemia and past history of cerebrovascular disease (Table 5). By the same method, DWMH score was

Table 3 Periventricular hyperintensity and deep white matter hyperintensity scores as determinants of cognitive impairment and low vitality

	PVH score			DWMH score		
	OR	95% CI	*P*-value	OR	95% CI	*P*-value
Cognitive impairment	1.185	1.084–1.295	<0.001	1.010	1.001–1.021	<0.05
Low vitality	1.260	1.133–1.401	<0.0001	1.025	1.012–1.039	<0.001

Cognitive impairment and low vitality were defined as MMSE ≤23 and vitality index ≤9, respectively. Multiple logistic analysis was performed after adjustment for age, sex, hypertension, diabetes, hyperlipidemia, and past history of cerebrovascular disease, of which all variables other than age were treated as categorical data. CI, confidence interval; DVMH, deep white matter hyperintensity; OR, odds ratio; PVH, periventricular hyperintensity.

significantly greater in subjects who exhibited the following symptoms of geriatric syndrome: hallucinations, delusions, gait disturbance, tripping, falls, pollakiuria, urinary incontinence and constipation. Multiple logistic analysis revealed that DWMH score remained a significant determinant of hallucinations, delusions, tripping, urinary incontinence and constipation after adjustment for age, sex, presence of hypertension, diabetes, hyperlipidemia and past history of cerebrovascular disease (Table 6).

Discussion

Elderly persons are affected by multiple chronic diseases. Once they are affected by serious illness, full recovery cannot be expected with medical treatment, because elderly patients are often trapped in a vicious circle of illness and poor quality of life (QOL). This is the reason why care and welfare contribute to the total well-being of the elderly. Physicians need to pay great attention to improving QOL as well as treating illness. Thus, it is important to comprehend the whole picture of their life by means of comprehensive geriatric assessment, which evaluates multiple aspects of an elderly person's life, such as activities of daily living, cognition, mood, vitality, communication and social environment.

The present study confirmed a negative correlation between the severity of WML and MMSE score. Multivariate analysis showed that the presence of WML was a significant risk factor for cognitive impairment, even after adjustment for confounding factors of age, sex, hypertension, diabetes, hyperlipidemia and past history of cerebrovascular disease. The mechanism and the size and location of WML that impair cognitive function are not yet clear. However, from previous studies, it seems convincing that a reduction of blood flow in the frontal lobe plays an important role in cognitive impairment in elderly people who exhibit WML.[20,21] Clinical manifestations of WML include attention deficit and a decline in information-processing ability.[4,13,22] Junque *et al.* reported the reappearance of primitive reflexes, one of the symptoms of frontal lobe dysfunction, in patients with WML.[11] In this study, patients with PVH showed attention deficit (incapability of calculation) and verbal inarticulacy (naming less vegetables), implying the impairment of frontal lobe function. WML, as reported previously,[6,23] were negatively correlated with vitality. Multiple logistic regression analysis, using potential risk factors including advanced age as confounding variables, found that the presence of WML was an independent risk factor for low vitality. Additionally, a relation between PVH score and apathy, a significant symptom of geriatric syndrome, was also found. From previous studies showing the importance of frontal lobe function in vitality,[24–26] we assume that blood flow reduction in the frontal lobe may account for the apathy and low vitality in patients with WML. More precisely, WML disrupting the frontal-subcortical circuit may result in dysfunction in the anterior cingulate and dorsolateral prefrontal circuits, thereby leading to apathy and decreased vitality.[5,6,20] Increase in PVH score or DWMH score was not apparently correlated with depression, probably because depression is associated with many factors such as aging, female sex, hyperlipidemia and medication.[27–29] The subjects in this study were mostly elderly (88.1%) and female (74.0%). We assume that these confounding conditions made it difficult to prove a true relation between WML and depression. From analysis of the association of WML with geriatric syndrome, it appears that WML have a relation to psychiatric symptoms (hallucinations and delusions), gait abnormalities (gait disturbance, tripping and falls), urinary symptoms (pollakiuria and urinary incontinence) and possibly with parkinsonism (swallowing difficulty, tremor and muscle stiffness). It was reported that WML were related to gait abnormalities,[5–7] presumably caused by disruption of the frontal-subcortical circuit.[30] Some other studies suggested that parkinsonism is also a contributing factor to gait disturbance in patients with WML.[6,31] Interestingly, we found that both gait abnormalities and symptoms of parkinsonism were associated with WML.

The present study confirmed an association between WML and voiding dysfunction (pollakiuria and incontinence). It was reported that urinary dysfunction was derived from damage to the frontal-subcortical

Table 4 Comparison of periventricular hyperintensity and deep white matter hyperintensity scores between subjects who did or did not exhibit each symptom of geriatric syndrome

Geriatric syndrome	Prevalence (%)	PVH score Symptom Present	Absent	*P*-value	DWMH score Symptom Present	Absent	*P*-value
Hallucination	6.8	**8.5 ± 5.9**	**4.4 ± 4.7**	**<0.01**	**59.8 ± 43.9**	**28.6 ± 35.4**	**<0.01**
Delusion	9.5	**7.6 ± 5.2**	**4.4 ± 4.8**	**0.01**	**56.1 ± 37.6**	**28.2 ± 35.9**	**<0.01**
Insomnia	18.9	4.2 ± 3.6	4.7 ± 4.9	0.56	31.4 ± 36.0	31.3 ± 37.6	0.98
Vertigo	18.9	6.1 ± 6.5	4.4 ± 4.4	0.06	33.4 ± 38.1	30.7 ± 37.0	0.70
Paralysis	2.1	8.5 ± 4.8	4.6 ± 4.9	0.12	59.5 ± 47.2	30.1 ± 36.3	0.11
Numbness	16.6	5.1 ± 4.6	4.6 ± 4.8	0.62	34.6 ± 40.0	29.9 ± 36,0	0.52
Gait disturbance	23.2	**6.7 ± 5.1**	**4.2 ± 4.7**	**<0.01**	**43.3 ± 41.7**	**27.5 ± 34.9**	**0.01**
Tripping	32.1	**6.4 ± 4.5**	**3.9 ± 4.9**	**<0.01**	**42.1 ± 43.7**	**25.9 ± 32.4**	**<0.01**
Falls	17.9	**6.6 ± 4.9**	**4.3 ± 4.8**	**0.01**	**45.8 ± 43.1**	**28.0 ± 35.0**	**0.01**
Pollakiuria	22.1	**8.0 ± 5.8**	**3.8 ± 4.2**	**<0.01**	**41.5 ± 41.0**	**41.5 ± 41.0**	**0.04**
Urinary incontinence	13.8	**7.5 ± 5.1**	**4.3 ± 4.8**	**<0.01**	**52.4 ± 44.9**	**52.4 ± 44.9**	**<0.01**
Constipation	26.3	5.8 ± 4.3	4.4 ± 5.1	0.08	**44.5 ± 45.1**	**44.5 ± 45.1**	**<0.01**
Decreased appetite	14.7	6.1 ± 4.4	4.5 ± 5.0	0.12	42.1 ± 42.6	42.1 ± 42.6	0.11
Weight loss	14.2	**6.9 ± 4.1**	**4.4 ± 5.0**	**0.01**	40.7 ± 41.3	40.7 ± 41.3	0.15
Apathy	7.6	**7.4 ± 3.6**	**4.4 ± 5.0**	**0.03**	30.7 ± 28.1	30.7 ± 28.1	0.97
Speech impairment	2.7	5.6 ± 5.2	4.5 ± 4.7	0.62	35.3 ± 48.0	35.3 ± 48.0	0.80
Swallowing difficulty	14.7	**12.2 ± 4.4**	**4.5 ± 4.8**	**<0.01**	44.6 ± 34.6	44.6 ± 34.6	0.40
Tremor	5.3	**9.1 ± 6.5**	**4.4 ± 4.7**	**<0.01**	45.0 ± 38.1	45.0 ± 38.1	0.24
Muscle stiffness	3.2	**9.2 ± 4.8**	**4.5 ± 4.9**	**0.02**	48.7 ± 43.4	48.7 ± 43.4	0.23

PVH and DWMH score are shown as mean ± SD. Boldface values are statistically significant ($P < 0.05$ by Student's *t*-test). DVMH, deep white matter hyperintensity; PVH, periventricular hyperintensity.

Table 5 Periventricular hyperintensity score as determinant of geriatric syndrome

	OR	*P*-value	95% CI
Hallucination	1.12	0.043	1.004–1.248
Tripping	1.11	0.005	1.032–1.194
Pollakiuria	1.17	0.001	1.067–1.278
Urinary incontinence	1.11	0.022	1.015–1.207
Weight loss	1.14	0.007	1.036–1.246
Apathy	1.14	0.027	1.015–1.276
Swallowing difficulty	1.35	0.019	1.050–1.741

Multiple logistic analysis was performed to analyze each symptom of geriatric syndrome, with adjustment for age, sex, hypertension, diabetes, hyperlipidemia and past history of cerebrovascular disease, of which all variables other than age were treated as categorical data. CI, confidence interval; OR, odds ratio.

Table 6 Deep white matter hyperintensity score as determinant of geriatric syndrome

	OR	*P*-value	95% CI
Hallucination	1.017	0.020	1.003–1.032
Delusion	1.016	0.024	1.002–1.030
Tripping	1.011	0.020	1.002–1.020
Urinary incontinence	1.016	0.008	1.004–1.028
Constipation	1.011	0.025	1.001–1.021

Multiple logistic analysis was performed to analyze each symptom of geriatric syndrome, with adjustment for age, sex, hypertension, diabetes, hyperlipidemia and past history of cerebrovascular disease, of which all variables other than age were treated as categorical data. CI, confidence interval; OR, odds ratio.

circuit.[5,20] In relation to the symptoms of parkinsonism (swallowing difficulty, tremor and muscle stiffness), this association was previously explained by dysfunction of the frontal-subcortical circuit.[4,31] The importance of this lesion was also suggested by a study showing that swallowing difficulty occurs with dysfunction of internuncial neurons that link the brainstem to the cerebral cortex.[32]

Considering the cause of manifestation of geriatric syndrome in patients with WML, it appears that damage to associative pathways in the frontal and subcortical regions due to ischemic hypoperfusion is an important mechanism.[5,20,21] It is necessary to localize the responsible connecting pathway for each symptom by a sophisticated approach in the future.

In conclusion, we showed that WML were associated with cognitive impairment, low vitality and geriatric syndrome of psychological disorders, gait disturbance,

urinary problems and parkinsonism. Evaluating WML in relation to geriatric syndrome and building a preventive measure against WML is an important future task for maintaining the independence of elderly people.

Acknowledgments

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References

1 Breteler MM, van Swieten JC, Bots ML *et al.* Cerebral white matter lesions, vascular risk factors, and cognitive function in a population-based study: the Rotterdam Study. *Neurology* 1994; **44**: 1246–1252.
2 Hachinski VC, Potter P, Merskey H. Leuko-araiosis. *Arch Neurol* 1987; **44**: 21–23.
3 Hunt AL, Orrison WW, Yeo RA *et al.* Clinical significance of MRI white matter lesions in the elderly. *Neurology* 1989; **39**: 1470–1474.
4 de Groot JC, de Leeuw FE, Oudkerk M *et al.* Cerebral white matter lesions and cognitive function: the Rotterdam Scan Study. *Ann Neurol* 2000; **47**: 145–151.
5 Kuo HK, Lipsitz LA. Cerebral white matter changes and geriatric syndromes: is there a link? *J Gerontol A Biol Sci Med Sci* 2004; **59**: 818–826.
6 Starkstein SE, Sabe L, Vazquez S *et al.* Neuropsychological, psychiatric, and cerebral perfusion correlates of leuko-araiosis in Alzheimer's disease. *J Neurol Neurosurg Psychiatry* 1997; **63**: 66–73.
7 Baloh RW, Ying SH, Jacobson KM. A longitudinal study of gait and balance dysfunction in normal older people. *Arch Neurol* 2003; **60**: 835–839.
8 Sakakibara R, Hattori T, Uchiyama T, Yamanishi T. Urinary function in elderly people with and without leukoaraiosis: relation to cognitive and gait function. *J Neurol Neurosurg Psychiatry* 1999; **67**: 658–660.
9 Tarvonen-Schroder S, Roytta M, Raiha I, Kurki T, Rajala T, Sourander L. Clinical features of leuko-araiosis. *J Neurol Neurosurg Psychiatry* 1996; **60**: 431–436.
10 Pantoni L, Garcia JH. The significance of cerebral white matter abnormalities 100 years after Binswanger's report. *Stroke* 1995; **26**: 1293–1301.
11 Junque C, Pujol J, Vendrell P *et al.* Leuko-araiosis on magnetic resonance imaging and speed of mental processing. *Arch Neurol* 1990; **47**: 151–156.
12 Fazekas F. Magnetic resonance signal abnormalities in asymptomatic individuals: their incidence and functional correlates. *Eur Neurol* 1989; **29**: 164–168.
13 Ylikoski R, Ylikoski A, Erkinjuntti T, Sulkava R, Raininko R, Tilvis R. White matter changes in healthy elderly persons correlate with attention and speed of mental processing. *Arch Neurol* 1993; **50**: 818–824.
14 Fu JH, Lu CZ, Hong Z, Dong Q, Luo Y, Wong KS. Extent of white matter lesions is related to acute subcortical infarcts and predicts further stroke risk in patients with first ever ischaemic stroke. *J Neurol Neurosurg Psychiatry* 2005; **76**: 793–796.
15 Taylor WD, MacFall JR, Provenzale JM *et al.* Serial MR imaging of volumes of hyperintense white matter lesions in elderly patients: correlation with vascular risk factors. *Am J Roentgenol* 2003; **181**: 571–576.
16 Folstein MF, Folstein SE, McHugh PR. "Mini-Mental State". A practical method for grading the cognitive state of patients for the clinician. *J Psychiat Res* 1975; **12**: 189–198.
17 Sheikh JI, Yesavage JA. Geriatric Depression Scale (GDS): recent evidence and development of a short version. *Clin Gerontol* 1986; **56**: 165–173.
18 Toba K, Nakai R, Akishita M *et al.* Vitality index as a useful tool to assess elderly with dementia. *Geriatr Gerontol Int* 2002; **2**: 23–29.
19 Cullen B, Fahy S, Cunningham CJ *et al.* Screening for dementia in an Irish community sample using MMSE: a comparison of norm-adjusted versus fixed cut-points. *Int J Geriatr Psychiatry* 2005; **20**: 371–376.
20 Pugh KG, Lipsitz LA. The microvascular frontal–subcortical syndrome of aging. *Neurobiol Aging* 2002; **23**: 421–431.
21 Yao H, Sadoshima S, Kuwabara Y, Ichiya Y, Fujishima M. Cerebral blood flow and oxygen metabolism in patients with vascular dementia of the Binswanger type. *Stroke* 1990; **21**: 1694–1699.
22 Burton EJ, Kenny RA, O'Brien J *et al.* White matter hyperintensities are associated with impairment of memory, attention, and global cognitive performance in older stroke patients. *Stroke* 2004; **35**: 1270–1275.
23 Thomas P, Hazif-Thomas C, Saccardy F, Vandermarq P. Loss of motivation and frontal dysfunction. Role of the white matter change. *Encephale* 2004; **30**: 52–59.
24 Okada K, Kobayashi S, Yamagata S, Takahashi K, Yamaguchi S. Poststroke apathy and regional cerebral blood flow. *Stroke* 1997; **28**: 2437–2441.
25 Craig AH, Cummings JL, Fairbanks L *et al.* Cerebral blood flow correlates of apathy in Alzheimer disease. *Arch Neurol* 1996; **53**: 1116–1120.
26 Benoit M, Koulibaly PM, Migneco O, Darcourt J, Pringuey DJ, Robert PH. Brain perfusion in Alzheimer's disease with and without apathy: a SPECT study with statistical parametric mapping analysis. *Psychiatry Res* 2002; **15**: 103–111.
27 Stordal E, Mykletun A, Dahl AA. The association between age and depression in the general population: a multivariate examination. *Acta Psychiatr Scand* 2003; **107**: 132–141.
28 Terao T, Iwata N, Kanazawa K *et al.* Low serum cholesterol levels and depressive state in human dock visitors. *Acta Psychiatr Scand* 2000; **101**: 231–234.
29 Noble RE. Depression in women. *Metabolism* 2005; **54**: 49–52.
30 Hennerici MG, Oster M, Cohen S, Schwartz A, Motsch L, Daffertshofer M. Are gait disturbances and white matter degeneration early indicators of vascular dementia? *Dementia* 1994; **5**: 197–202.
31 Piccini P, Pavese N, Canapicchi R *et al.* White matter hyperintensities in Parkinson's disease. Clinical correlations. *Arch Neurol* 1995; **52**: 191–194.
32 Daniels SK, Foundas AL. Lesion localization in acute stroke patients with risk of aspiration. *J Neuroimaging* 1999; **9**: 91–98.

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Adiponectin Antagonizes Stimulatory Effect of Tumor Necrosis Factor-α on Vascular Smooth Muscle Cell Calcification: Regulation of Growth Arrest-Specific Gene 6-Mediated Survival Pathway by Adenosine 5′-Monophosphate-Activated Protein Kinase

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Adiponectin exhibits diverse protective effects against atherogenesis and antagonizes many effects of TNFα. Here, we investigated the effect of adiponectin and TNFα on vascular calcification, a critical event in the development and progression of vascular disease. In human aortic smooth muscle cells (HASMC), TNFα augmented inorganic phosphate (Pi)-induced calcification, whereas adiponectin significantly suppressed it and abolished the stimulatory effect of TNFα in a concentration-dependent manner. Similarly, adiponectin ameliorated the accelerating effect of TNFα on Pi-induced apoptosis, the essential process of HASMC calcification. Furthermore, these effects of TNFα and adiponectin were associated with AMP-activated protein kinase (AMPK)-dependent growth arrest-specific gene 6 (Gas6) expression and Akt signaling. The AMPK activator, 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), induced phosphorylation of AMPK and significantly inhibited Pi-induced calcification in HASMC. Conversely, pharmacological inhibition of AMPK by compound C blocked both AMPK activation and the inhibitory effect of adiponectin on calcification, providing evidence that AMPK plays a regulatory role in vascular calcification. Reporter assay revealed that adiponectin restored Gas6 promoter activity decreased by TNFα, and the effect of adiponectin was abrogated by compound C. These results demonstrate that adiponectin antagonizes the stimulatory effect of TNFα on vascular calcification by restoration of the AMPK-dependent Gas6-mediated survival pathway. (*Endocrinology* 149: 1646–1653, 2008)

VASCULAR CALCIFICATION is often encountered in advanced atherosclerotic lesions and is a common consequence of aging (1, 2). Calcification of the coronary arteries has been shown to be positively correlated with atherosclerotic plaque burden, increased risk of myocardial infarction, and plaque instability (3–5). We recently demonstrated that apoptosis plays an important role in inorganic phosphate (Pi)-induced vascular smooth muscle cell (VSMC) calcification (6). This type of calcification is dependent on down-regulation of the growth arrest-specific gene 6 (Gas6)-mediated survival pathway.

Adiponectin is an adipocyte-derived cytokine that exhibits protective properties in the heart and blood vessels (7–10). It accumulates in injured arteries from plasma and suppresses the endothelial inflammatory response (11) and VSMC proliferation (12). Furthermore, low plasma adiponectin levels are associated with progression of coronary artery calcification in type 1 diabetic and nondiabetic subjects, independent of other cardiovascular risk factors (13). Experimental studies have shown that adiponectin reduces TNFα production in response to various stresses, whereas TNFα attenuates adiponectin production, resulting in a reduction of plasma adiponectin levels (14–16). In addition to the inverse relationship between their expression, increasing evidence supports suppressive effects on each other's function (11, 17, 18). Given the importance of the reciprocal effects of TNFα and adiponectin, it is not clear whether both play a regulatory role in VSMC calcification.

Most of the beneficial actions of adiponectin are accounted for by the activation of AMP-activated protein kinase (AMPK) (19, 20). AMPK is a serine/threonine protein kinase that plays a key role in metabolic homeostasis in all eukaryotic cell types (21). Cardioprotective effects of adiponectin, including antiapoptotic actions, are also likely to be dependent on AMPK (19, 22, 23). However, the role of AMPK in the effect of adiponectin on VSMC calcification has not been addressed.

In the present study, we investigated whether adiponectin and TNFα modulate Pi-induced VSMC calcification by regulating apoptosis. We found that TNFα had a stimulatory effect, whereas adiponectin had an inhibitory effect on Pi-induced apoptosis and calcification in human aortic smooth muscle cells (HASMC). Furthermore, these actions were mediated by regulation of Gas6 at the transcription level via AMPK activation.

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Abbreviations: AICAR, 5-Aminoimidazole-4-carboxamide ribonucleoside; AMPK, AMP-activated protein kinase; Gas6, growth arrest-specific gene 6; HASMC, human aortic smooth muscle cells; Pi, inorganic phosphate; PP2C, protein phosphatase 2C; siRNA, small interfering RNA; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling; VSMC, vascular smooth muscle cells.

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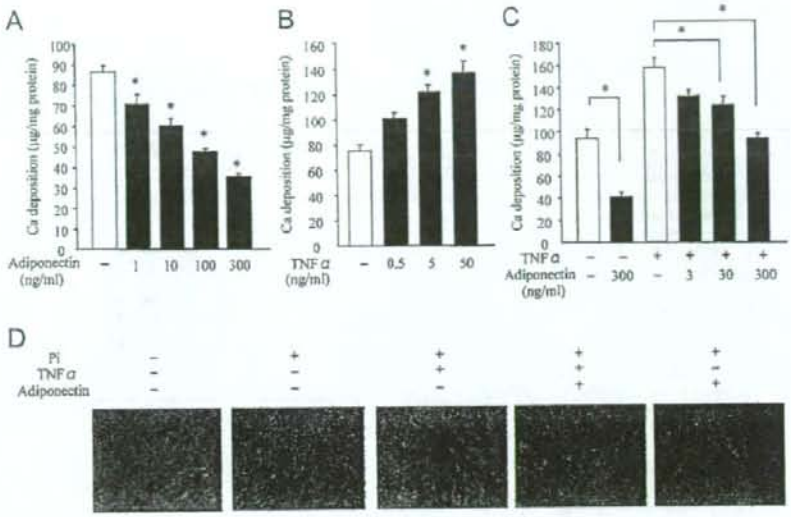

FIG. 1. Effect of adiponectin and TNFα on Pi-induced calcification. A and B, HASMC were cultured with the indicated concentrations of adiponectin (A) or TNFα (B) in calcification medium. They were added simultaneously when the medium was changed every 2 d. C, The effect of TNFα (20 ng/ml) and adiponectin with the indicated concentrations on Ca deposition was determined at 6 d. D, The effect of TNFα (20 ng/ml) and adiponectin (300 ng/ml) on Ca deposition was evaluated with von Kossa's staining at the light microscopic level. All values are presented as mean ± SE (n = 6). *, $P < 0.05$ by Bonferroni test. Each experiment was performed at least in triplicate for each condition.

Materials and Methods

Cell culture

HASMC were purchased from Clonetics Corp. (San Diego, CA). They were cultured in DMEM supplemented with 20% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37 C in a humidified atmosphere with 5% CO_2. HASMC were used up to passage 8 for the experiments.

Induction and quantification of calcification

For Pi-induced calcification, Pi (a mixed solution of Na_2HPO_4 and NaH_2PO_4 whose pH was adjusted to 7.4) was added to serum-supplemented DMEM to a final concentration of 2.6 mM (calcification medium). Ca deposition was evaluated by the *o*-cresolphthalein complexone method (C-Test; WAKO, Osaka, Japan) and von Kossa's staining, as previously described (6, 24).

Determination of apoptosis

To examine the effect of TNFα (Sigma-Aldrich, St. Louis, MO) and adiponectin (R&D Systems, Minneapolis, MN) on Pi-induced apoptosis, they were added simultaneously when the medium was switched to the calcification medium. Apoptosis was detected by DNA fragmentation with a cell-death detection ELISA^{plus} kit (Roche, Manheim, Germany) and ter-

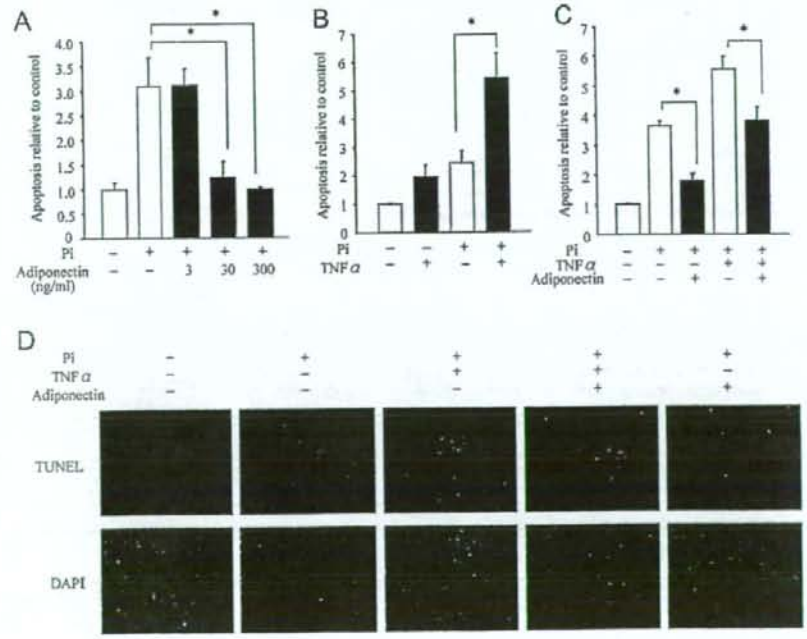

FIG. 2. Effect of adiponectin and TNFα on Pi-induced apoptosis. HASMC were cultured with the indicated concentrations of adiponectin for 6 d. Calcification medium was exchanged every 2 d. A, A quantitative index of apoptosis, determined by ELISA, is presented as the value relative to that without Pi treatment. B, HASMC were incubated with or without TNFα (20 ng/ml) in the absence or presence of 2.6 mM Pi for 6 d. C and D, On d 6, the effect of adiponectin (300 ng/ml) and TNFα (20 ng/ml) on apoptosis in calcification medium was determined by ELISA (C) and evaluated with TUNEL staining (D, *green*). Nuclei were counterstained with DAPI (*blue*). All values are presented as mean ± SE (n = 3). *, $P < 0.05$ by Bonferroni test. Each experiment was performed in triplicate for each condition.