

that was decreased by TNF α . Indeed, suppression of AMPK by compound C clearly abrogated this beneficial effect of adiponectin. This result suggests that AMPK participates in the transcriptional regulation of Gas6 by adiponectin and TNF α . Several studies support that AMPK regulates the expression of particular genes at the transcriptional level (37–39). For example, AMPK activation by AICAR enhanced activator protein 1-mediated proopiomelanocortin promoter activities, which were completely abolished by compound C (37). AMPK has been shown to mediate the transcription signal that leads to the repression of phosphoenolpyruvate carboxykinase expression, a key enzyme of gluconeogenesis, through phosphorylation of a transcription factor, AICAR-responsive element binding protein (38). It has also been observed that AICAR treatment is able to reduce nuclear factor- κ B-regulated transcription, which is activated by TNF α (39).

Consistent with our findings, it has been recently reported that TNF α directly suppresses AMPK activation by promoting protein phosphatase 2C (PP2C) activity via TNF receptor-1 (40). PP2C has been proposed as one of modulators of the covalent regulation of AMPK (41). Increased PP2C levels account for the reduced AMPK activity and phosphorylation after TNF α treatment (40). On the other hand, LKB1 [also known as serine/threonine kinase II (STK II)] is the well-known, principal upstream kinase of AMPK (42, 43) that is regulated by adiponectin (44). AMPK activation by adiponectin is considered to be mediated by the cell surface receptors adiponectin receptors 1 and 2 (45). Another adiponectin receptor, T-cadherin, has recently been identified (46). In preliminary experiments, we found that all of the three adiponectin receptors were endogenously expressed in HASMC, and Pi did not affect their expression (data not shown). Taking these observations together, we hypothesized the mechanism of regulation by adiponectin and TNF α on Pi-induced vascular calcification (Fig. 7). However, further intensive investigations are required to elucidate the role of each player in VSMC calcification.

In summary, adiponectin inhibited VSMC calcification and antagonized the stimulatory effect of TNF α . This action was caused by preventing apoptosis via AMPK activation, followed by restoration of the Gas6-mediated survival pathway. AMPK regulated Gas6 expression at the transcriptional level. AMPK activation regulated by adiponectin and TNF α in vascular calcification might be a key to the management of cardiovascular disease.

Acknowledgments

We thank Yuki Ito for technical assistance.

Received July 25, 2007. Accepted December 26, 2007.

Address all correspondence and requests for reprints to: Masahiro Akishita, M.D., Ph.D., Department of Geriatric Medicine, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. E-mail: akishita-tky@umin.ac.jp.

This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan (No. 18590801 and No. 19590854), Novartis Foundation for Gerontological Research, Kanzawa Medical Research Foundation, Ono Medical Research Foundation, and Takeda Research Foundation.

Disclosure Statement: The authors have nothing to disclose.

References

- Wexler L, Brundage B, Crouse J, Detrano R, Fuster V, Maddahi J, Rumberger J, Stanford W, White R, Taubert K 1996 Coronary artery calcification: pathophysiology, epidemiology, imaging methods, and clinical implications. A statement for health professionals from the American Heart Association Writing Group. *Circulation* 94:1175–1192
- Johnson RC, Leopold JA, Loscalzo J 2006 Vascular calcification: pathobiological mechanisms and clinical implications. *Circ Res* 99:1044–1059
- van Poelje NM, Mattace-Raso FU, Vlietghart R, Grobbee DE, Asmar R, van der Kuip DA, Hofman A, de Feijter BJ, Oudkerk M, Witteman JC 2006 Aortic stiffness is associated with atherosclerosis of the coronary arteries in older adults: the Rotterdam Study. *J Hypertens* 24:2371–2376
- Arad Y, Spadaro LA, Goodman K, Newstein D, Guerci AD 2000 Prediction of coronary events with electron beam computed tomography. *J Am Coll Cardiol* 36:1253–1260
- Thompson GR, Partridge J 2004 Coronary calcification score: the coronary-risk impact factor. *Lancet* 363:557–559
- Son BK, Kozaki K, Iijima K, Eto M, Kojima T, Ota H, Senda Y, Maemura K, Nakano T, Akishita M, Ouchi Y 2006 Statins protect human aortic smooth muscle cells from inorganic phosphate-induced calcification by restoring Gas6-Axl survival pathway. *Circ Res* 98:1024–1031
- Nakamura Y, Shimada K, Fukuda D, Shimada Y, Ehara S, Hirose M, Kataoka T, Kamimori K, Shimodozono S, Kobayashi Y, Yoshiyama M, Takeuchi K, Yoshikawa J 2004 Implications of plasma concentrations of adiponectin in patients with coronary artery disease. *Heart* 90:528–533
- Fischon T, Girman CJ, Hotamisligil GS, Rifai N, Hu FB 2004 Plasma adiponectin levels and risk of myocardial infarction in men. *JAMA* 291:1730–1737
- Schulze MB, Shai I, Rimm EB, Li T, Rifai N, Hu FB 2005 Adiponectin and future coronary heart disease events among men with type 2 diabetes. *Diabetes* 54:534–539
- Kojima S, Funahashi T, Sakamoto T, Miyamoto S, Seoijima H, Hokamaki J, Kajiwara I, Sugiyama S, Yoshimura M, Fujimoto K, Miyao Y, Suefuji H, Kitagawa A, Ouchi N, Kihara S, Matsuzawa Y, Ogawa H 2003 The variation of plasma concentrations of a novel, adipocyte-derived protein, adiponectin, in patients with acute myocardial infarction. *Heart* 89:667
- Ouchi N, Kihara S, Arita Y, Maeda K, Kuriyama H, Okamoto Y, Hotta K, Nishida M, Takahashi M, Nakamura T, Yamashita S, Funahashi T, Matsuzawa Y 1999 Novel modulator for endothelial adhesion molecules: adipocyte-derived plasma protein adiponectin. *Circulation* 100:2473–2476
- Arita Y, Kihara S, Ouchi N, Maeda K, Kuriyama H, Okamoto Y, Kumada M, Hotta K, Nishida M, Takahashi M, Nakamura T, Shimomura I, Muraguchi M, Ohmoto Y, Funahashi T, Matsuzawa Y 2002 Adipocyte-derived plasma protein adiponectin acts as a platelet-derived growth factor-BB-binding protein and regulates growth factor-induced common postreceptor signal in vascular smooth muscle cell. *Circulation* 105:2893–2898
- Maahs DM, Ogen LG, Kinney GL, Wadwa P, Snell-Bergeon JK, Dabelea D, Hokanson JE, Ehrlich J, Eckel RH, Rewers M 2005 Low plasma adiponectin levels predict progression of coronary artery calcification. *Circulation* 111:747–753
- Ouchi N, Kihara S, Arita Y, Nishida M, Matsuyama A, Okamoto Y, Ishigami M, Kuriyama H, Kishida K, Nishizawa H, Hotta K, Muraguchi M, Ohmoto Y, Yamashita S, Funahashi T, Matsuzawa Y 2001 Adipocyte-derived plasma protein, adiponectin, suppresses lipid accumulation and class A scavenger receptor expression in human monocyte-derived macrophages. *Circulation* 103:1057–1063
- Maeda N, Takahashi M, Funahashi T, Kihara S, Nishizawa H, Kishida K, Nagaretani H, Matsuda M, Komuro R, Ouchi N, Kuriyama H, Hotta K, Nakamura T, Shimomura I, Matsuzawa Y 2001 PPAR γ ligands increase expression and plasma concentrations of adiponectin, an adipose-derived protein. *Diabetes* 50:2094–2099
- Kern PA, Di Gregorio GB, Lu T, Rassouli N, Ranganathan G 2003 Adiponectin expression from human adipose tissue: relation to obesity, insulin resistance, and tumor necrosis factor- α expression. *Diabetes* 52:1779–1785
- Maeda N, Shimomura I, Kishida K, Nishizawa H, Matsuda M, Nagaretani H, Furuyama N, Kondo H, Takahashi M, Arita Y, Komuro R, Ouchi N, Kihara S, Tochino Y, Okutomi K, Horie M, Takeda S, Aoyama T, Funahashi T, Matsuzawa Y 2002 Diet-induced insulin resistance in mice lacking adiponectin/ACRP30. *Nat Med* 8:731–737
- Ouchi N, Kihara S, Arita Y, Okamoto Y, Maeda K, Kuriyama H, Hotta K, Nishida M, Takahashi M, Muraguchi M, Ohmoto Y, Nakamura T, Yamashita S, Funahashi T, Matsuzawa Y 2000 Adiponectin, an adipocyte-derived plasma protein, inhibits endothelial NF- κ B signaling through a cAMP-dependent pathway. *Circulation* 102:1296–1301
- Shibata R, Sato K, Pimentel DR, Takemura Y, Kihara S, Ohashi K, Funahashi T, Ouchi N, Walsh K 2005 Adiponectin protects against myocardial ischemia-reperfusion injury through AMPK- and COX-2-dependent mechanisms. *Nat Med* 11:1096–1103
- Ouchi N, Kobayashi H, Kihara S, Kumada M, Sato K, Inoue T, Funahashi T, Walsh K 2004 Adiponectin stimulates angiogenesis by promoting cross-talk

- between AMP-activated protein kinase and Akt signaling in endothelial cells. *J Biol Chem* 279:1304–1309
21. Kudo N, Barr AJ, Barr RL, Desai S, Lopaschuk GD 1995 High rates of fatty acid oxidation during reperfusion of ischemic hearts are associated with a decrease in malonyl-CoA levels due to an increase in 5'-AMP-activated protein kinase inhibition of acetyl-CoA carboxylase. *J Biol Chem* 270:17513–17520
 22. Ouchi N, Kihara S, Funahashi T, Matsuzawa Y, Walsh K 2003 Obesity, adiponectin and vascular inflammatory disease. *Curr Opin Lipidol* 14:561–566
 23. Kobayashi H, Ouchi N, Kihara S, Walsh K, Kumada M, Abe Y, Funahashi T, Matsuzawa Y 2004 Selective suppression of endothelial cell apoptosis by the high molecular weight form of adiponectin. *Circ Res* 94:e27–e31
 24. Son BK, Kozaki K, Iijima K, Eto M, Nakano T, Akishita M, Ouchi Y 2007 Gas6/Axl-PI3K/Akt pathway plays a central role in the effect of statins on inorganic phosphate-induced calcification of vascular smooth muscle cells. *Eur J Pharmacol* 556:1–8
 25. Corton JM, Gillespie JG, Hawley SA, Hardie DG 1995 5-Aminoimidazole-4-carboxamide ribonucleoside. A specific method for activating AMP-activated protein kinase in intact cells? *Eur J Biochem* 229:558–565
 26. Gualillo O, González-Juanatey JR, Lago F 2007 The emerging role of adipokines as mediators of cardiovascular function: physiologic and clinical perspectives. *Trends Cardiovasc Med* 17:275–283
 27. Inoue T, Kotooka N, Morooka T, Komoda H, Uchida T, Aso Y, Inukai T, Okuno T, Node K 2007 High molecular weight adiponectin as a predictor of long-term clinical outcome in patients with coronary artery disease. *Am J Cardiol* 100:569–574
 28. Berg AH, Combs TP, Scherer PE 2002 ACRP 30/adiponectin: an adipokine regulating glucose and lipid metabolism. *Trends Endocrinol Metab* 13:84–89
 29. Lin LY, Lin CY, Su TC, Liao CS 2004 Angiotensin II-induced apoptosis in human endothelial cells is inhibited by adiponectin through restoration of the association between endothelial nitric oxide synthase and heat shock protein 90. *FEBS Lett* 574:106–110
 30. Russell 3rd RR, Li J, Coven DL, Pypaert M, Zechner C, Palmeri M, Giordano FJ, Mu J, Birnbaum MJ, Young LH 2004 AMP-activated protein kinase mediates ischemic glucose uptake and prevents postischemic cardiac dysfunction, apoptosis, and injury. *J Clin Invest* 114:495–503
 31. Li J, Miller EJ, Ninomiya-Tsuji J, Russell 3rd RR, Young LH 2005 AMP-activated protein kinase activates p38 mitogen-activated protein kinase by increasing recruitment of p38 MAPK to TAB1 in the ischemic heart. *Circ Res* 97:872–879
 32. Marsin AS, Bertrand L, Rider MH, Deprez J, Beauloye C, Vincent MF, Van den Berghe G, Carling D, Hue L 2000 Phosphorylation and activation of heart PFK-2 by AMPK has a role in the stimulation of glycolysis during ischaemia. *Curr Biol* 10:1247–1255
 33. Kudo N, Gillespie JG, Kung L, Witters LA, Schulz R, Clanachan AS, Lopaschuk GD 1996 Characterization of 5'-AMP-activated protein kinase activity in the heart and its role in inhibiting acetyl-CoA carboxylase during reperfusion following ischemia. *Biochim Biophys Acta* 1301:67–75
 34. Makinde AO, Gamble J, Lopaschuk GD 1997 Upregulation of 5'-AMP-activated protein kinase is responsible for the increase in myocardial fatty acid oxidation rates following birth in the newborn rabbit. *Circ Res* 80:482–489
 35. Nagata D, Takeda R, Sata M, Satonaka H, Suzuki E, Nagano T, Hirata Y 2004 AMP-activated protein kinase inhibits angiotensin II-stimulated vascular smooth muscle proliferation. *Circulation* 110:444–451
 36. Igata M, Motoshima H, Tsuruzoe K, Kojima K, Matsumura T, Kondo T, Taguchi T, Nakamaru K, Yano M, Kukidome D, Matsumoto K, Toyonaga T, Asano T, Nishikawa T, Araki E 2005 Adenosine monophosphate-activated protein kinase suppresses vascular smooth muscle cell proliferation through the inhibition of cell cycle progression. *Circ Res* 97:837–844
 37. Iwasaki Y, Nishiyama M, Taguchi T, Kambayashi M, Asai M, Yoshida M, Nigawara T, Hashimoto K 2007 Activation of AMP-activated protein kinase stimulates proopiomelanocortin gene transcription in A1720 corticotroph cells. *Am J Physiol Endocrinol Metab* 292:E1899–E1905
 38. Inoue E, Yamauchi J 2006 AMP-activated protein kinase regulates PEPCCK gene expression by direct phosphorylation of a novel zinc finger transcription factor. *Biochem Biophys Res Commun* 351:793–799
 39. Solaz-Fuster MC, Gimeno-Alcaniz JV, Casado M, Sanz P 2006 TRIP6 transcriptional co-activator is a novel substrate of AMP-activated protein kinase. *Cell Signal* 18:1702–1712
 40. Steinberg GR, Michell BJ, van Denderen BJ, Watt MJ, Carey AL, Fam BC, Andrikopoulos S, Proietto J, Gürgün CZ, Carling D, Hotamisligil GS, Febbraio MA, Kay TW, Kemp BE 2006 Tumor necrosis factor α -induced skeletal muscle insulin resistance involves suppression of AMP-kinase signaling. *Cell Metab* 4:465–474
 41. Davies SP, Helps NR, Cohen PT, Hardie DG 1995 5'-AMP inhibits dephosphorylation, as well as promoting phosphorylation, of the AMP-activated protein kinase. Studies using bacterially expressed human protein phosphatase-2C α and native bovine protein phosphatase-2AC. *FEBS Lett* 377:421–425
 42. Hawley SA, Boudeau J, Reid JL, Mustard KJ, Udd L, Mäkelä TP, Alessi DR, Hardie DG 2003 Complexes between the LKB1 tumor suppressor, STRAD α/β and MO25 α/β are upstream kinases in the AMP-activated protein kinase cascade. *J Biol Chem* 278:12181–12188
 43. Woods A, Johnstone SR, Dickerson K, Leiper FC, Fryer LG, Neumann D, Schlattner U, Wallimann T, Carlson M, Carling D 2003 LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. *Curr Biol* 13:2004–2008
 44. Imai K, Inukai K, Ikegami Y, Awata T, Katayama S 2006 LKB1, an upstream AMPK kinase, regulates glucose and lipid metabolism in cultured liver and muscle cells. *Biochem Biophys Res Commun* 351:595–601
 45. Yamauchi T, Kamon J, Ito Y, Tsuchida A, Yokomizo T, Kita S, Sugiyama T, Miyagishi M, Hara K, Tsunoda M, Murakami K, Ohteki T, Uchida S, Takekawa S, Waki H, Tsuno NH, Shibata Y, Terauchi Y, Froguel P, Tobe K, Koyasu S, Taira K, Kitamura T, Shimizu T, Nagai R, Kadowaki T 2003 Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. *Nature* 423:762–769
 46. Hug C, Wang J, Ahmad NS, Bogan JS, Tsao TS, Lodish HF 2004 T-cadherin is a receptor for hexameric and high-molecular-weight forms of ACRP30/adiponectin. *Proc Natl Acad Sci USA* 101:10308–10313

Endocrinology is published monthly by The Endocrine Society (<http://www.endo-society.org>), the foremost professional society serving the endocrine community.

Association of Plasma Dehydroepiandrosterone-Sulfate Levels with Endothelial Function in Postmenopausal Women with Coronary Risk Factors

Masahiro AKISHITA¹⁾, Masayoshi HASHIMOTO²⁾, Yumiko OHIKE¹⁾,
Sumito OGAWA¹⁾, Katsuya IJIMA¹⁾, Masato ETO¹⁾, and Yasuyoshi OUCHI¹⁾

Age-related decline of plasma dehydroepiandrosterone-sulfate (DHEA-S) levels may be associated with the risk of cardiovascular disease in women. We investigated whether plasma DHEA-S levels are related to endothelial function in postmenopausal women with coronary risk factors. One hundred and fifteen postmenopausal women (mean age \pm SD: 57 \pm 5 years; range: 48–65 years) who underwent measurement of flow-mediated vasodilation (FMD) of the brachial artery using ultrasonography were enrolled. Plasma hormone levels were determined in the morning after a 14-h fast, and the relationship between hormone levels and FMD was analyzed. DHEA-S was significantly correlated with %FMD ($r=0.392$, $p<0.001$), while estradiol, total testosterone and cortisol were not. %FMD in the highest quartile of DHEA-S was 1.8-fold higher than that in the lowest quartile (5.3 ± 1.3 vs. 2.9 ± 2.0 [means \pm SD], $p<0.01$). Multiple regression analysis revealed that DHEA-S was related to %FMD independent of age, body mass index, hypertension, hyperlipidemia, diabetes mellitus and smoking ($\beta=0.344$, $p<0.01$), and was itself independent of age, body mass index, systolic blood pressure, total cholesterol, high-density lipoprotein (HDL) cholesterol, fasting plasma glucose and smoking ($\beta=0.291$, $p<0.05$). In conclusion, plasma DHEA-S levels were weakly but significantly related to endothelial function in postmenopausal women independent of other coronary risk factors, suggesting a protective effect of DHEA on the endothelium. (*Hypertens Res* 2008; 31: 69–74)

Key Words: endothelium, vasodilation, risk factor, man, nitric oxide

Introduction

Plasma levels of dehydroepiandrosterone-sulfate (DHEA-S), the most abundant circulating steroid that is secreted from the adrenal cortex, decline with advancing age in men and women (1, 2). The age-related decrease in DHEA-S concentrations has often been associated with the pathological processes of aging, such as osteoporosis, depression and

dementia (3, 4). A number of studies have investigated the link between DHEA-S and cardiovascular disease, though with inconsistent results (5–7). Among them, Haffner *et al.* have shown that low DHEA-S levels predicted ischemic heart disease mortality in diabetic women (8). The association of low DHEA-S levels with carotid artery atherosclerosis (9), obesity (10) and decreased diurnal blood pressure variability (11) also suggests a vasoprotective role of DHEA(-S) in women. Furthermore, experimental studies showing endothe-

From the ¹⁾Department of Geriatric Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan; and ²⁾Department of General Internal Medicine, Kobe University School of Medicine, Kobe, Japan.

This study was supported by Health and Labour Sciences Research Grants (H17-Choju-046) from the Ministry of Health, Labour and Welfare of Japan, by the Gerontology Small Research Grant Program from the Division of Project Coordination, The University of Tokyo and by grants from the NOVARTIS Foundation for Gerontological Research and the Yamaguchi Endocrine Research Association.

Address for Reprints: Masahiro Akishita, M.D., Ph.D., Department of Geriatric Medicine, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. E-mail: akishita-ky@umin.ac.jp

Received March 14, 2007; Accepted in revised form August 5, 2007.

Table 1. Characteristics of Study Subjects (n=115)

Age, years	57±5	[48–65]
Body mass index, kg/m ²	23.3±4.0	[17.6–35.0]
Risk factors		
Hypertension, n (%)	35 (30)	
Hyperlipidemia, n (%)	71 (62)	
Diabetes mellitus, n (%)	20 (17)	
Current smoker, n (%)	17 (15)	
Hemodynamic and vascular measurements		
Systolic blood pressure, mmHg	123±17	[93–170]
Diastolic blood pressure, mmHg	70±10	[52–100]
%FMD	4.8±2.4	[0.0–12.7]
%NTG	14.0±4.5	[4.1–22.5]
Carotid IMT, mm	0.92±0.22	[0.46–1.45]
Blood chemistry and hormones		
Total cholesterol, mmol/L	5.72±1.01	[3.73–8.96]
HDL cholesterol, mmol/L	1.66±0.42	[0.77–2.87]
Triglycerides, mmol/L	1.18±0.65	[0.36–3.49]
Fasting plasma glucose, mmol/L	5.42±1.22	[4.00–10.43]
Hemoglobin A1c, %	5.5±1.0	[4.3–9.6]
Estradiol, pmol/L	35±26	[18–160]
Testosterone, nmol/L	0.85±0.43	[0.21–2.01]
DHEA-S, μmol/L	2.28±1.07	[0.37–5.02]
Cortisol, nmol/L	316±121	[110–728]

Values except risk factors are expressed as mean±SD [range]. %FMD, percent flow-mediated dilation of brachial artery; %NTG, percent nitroglycerin-induced dilation of brachial artery; IMT, intima-media thickness of common carotid artery; HDL, high-density lipoprotein; DHEA-S, dehydroepiandrosterone-sulfate.

lium-dependent (12) and -independent (13) vasodilating effects of DHEA(-S) led us to hypothesize that postmenopausal women with low plasma DHEA-S levels would have impaired vasomotor function.

To test this hypothesis, we conducted a cross-sectional survey of 115 postmenopausal women by examining flow-mediated dilation (FMD) of the brachial artery and plasma sex hormones, and showed that low DHEA-S levels were associated with endothelial dysfunction.

Methods

Subjects

One hundred and fifteen postmenopausal women who underwent examination of vasomotor function of the brachial artery and intima-media thickness (IMT) of the carotid artery in our department were enrolled. The subjects were referred to our department to check their cardiovascular disease or risks. All of them were in chronic stable condition. A history was taken, and physical examination and laboratory tests were performed in all subjects. Subjects with a history of cardiovascu-

lar disease, including stroke, coronary heart disease, congestive heart failure and peripheral arterial disease, malignancy, overt endocrine disease or administration of steroid hormones, were excluded. The postmenopausal status of each subject was confirmed by the fact that at least 12 months had passed since her last menses and by the measurement of follicular stimulating hormone and estradiol. The characteristics of the study subjects are shown in Table 1.

Seventy-three percent of the subjects had one or more classical coronary risk factors, such as hypertension, hyperlipidemia, diabetes mellitus or current smoking. Hypertension, hyperlipidemia and diabetes mellitus were considered to be present based on the published diagnostic criteria (14–16) or if the subjects were taking any medications for these diseases. Eighty-six percent of the hypertensive subjects were treated: 75% with calcium antagonists, 19% with angiotensin-converting enzyme inhibitors, 10% with diuretics and 7% with β blockers. Eighty-six percent of the hyperlipidemic subjects were treated with statins, and 75% of the diabetic subjects were treated with oral hypoglycemic agents. None of the study subjects were taking nitrates. Each subject gave written informed consent before enrollment in this study. The study protocol was approved by the ethics committee of the Graduate School of Medicine, the University of Tokyo.

Vascular Measurement

Vasomotor function of the brachial artery was evaluated using an ultrasound machine according to the method described previously (17). Briefly, endothelium-dependent flow-mediated vasodilation (%FMD) was measured as the maximal percent change of the vessel diameter after reactive hyperemia. The subjects were examined in the morning after a 14-h overnight fast with no medication. Subsequently, endothelium-independent nitroglycerin-induced vasodilation (%NTG) was measured as the maximal percent change of the vessel diameter after sublingual administration of nitroglycerin spray (0.3 mg; Toa Eiyo Co., Tokyo). Carotid IMT was evaluated using an ultrasound machine as described previously (17).

Plasma Hormones

Blood sampling was performed in the morning of the vascular measurement after a 14-h overnight fast, and plasma was stored at -80°C until assay. Plasma estradiol, testosterone (total testosterone), DHEA-S and cortisol concentrations were determined using sensitive radioimmunoassays by a commercial laboratory (SRL Inc., Tokyo, Japan). The intra-assay coefficients of variation for these measurements were less than 5%.

Because plasma was deep-frozen for 3–7 years, we checked the change in titers using the stored samples, which had been measured at sampling 5–7 years before. The Pearson's correlation coefficients between the two measurements were 0.965

Table 2. Pearson's Correlation Coefficients between Age, Vascular Measurements and Plasma Hormones

	Age	%FMD	Carotid IMT
Estradiol	-0.108	0.041	-0.136
Testosterone	0.061	0.019	-0.088
DHEA-S	-0.198*	0.392†	-0.187*
Cortisol	0.074	0.140	0.064

%FMD, percent flow-mediated dilation of brachial artery; IMT, intima-media thickness of common carotid artery. † $p < 0.001$, * $p < 0.05$.

for estradiol ($n=34$), 0.976 for testosterone ($n=20$), 0.991 for DHEA-S ($n=15$) and 0.937 for cortisol ($n=16$), indicating that there was no significant change in plasma titers in our frozen samples.

Data Analysis

Pearson's simple correlation coefficients between age, vascular measurements and plasma hormones were determined. Standardized regression coefficients from multiple regression analysis of vascular measurements in relation to age, coronary risk factors and plasma hormones were determined. Differences between the groups were analyzed using one-factor ANOVA, followed by Newman-Keuls' test. A value of $p < 0.05$ was considered statistically significant. Statistical analyses were performed using the SPSS ver. 11.0 software.

Results

Changes in Plasma Hormones and Vascular Measurements According to Age and Coronary Risk Factors

Plasma levels of DHEA-S declined with age, while those of estradiol, testosterone and cortisol did not significantly change according to age (Table 2). %FMD decreased slightly but not significantly ($r = -0.166$, $p = 0.08$), while carotid IMT increased significantly ($r = 0.337$, $p < 0.01$) with advancing age.

The subjects with hypertension, hyperlipidemia or diabetes showed impaired %FMD compared to those without these diseases (hypertension, 4.0 ± 2.4 vs. 5.1 ± 2.2 ; hyperlipidemia, 4.3 ± 2.3 vs. 5.6 ± 2.2 ; diabetes mellitus, 4.0 ± 2.8 vs. 5.2 ± 2.2 ; $p < 0.05$). %FMD in the patients taking antihypertensive agents, statins or hypoglycemic agents was comparable to or smaller than that in the patients without medical agents (hypertension, 4.0 ± 2.5 vs. 4.3 ± 2.3 , n.s.; hyperlipidemia, 4.1 ± 2.2 vs. 5.1 ± 2.4 , n.s.; diabetes, 3.5 ± 2.7 vs. 6.5 ± 1.5 , $p < 0.05$), suggesting that the favorable effects of medical treatment on endothelial function, if present, might have been lost in patients with a long history of coronary risk factors. In contrast, none of the plasma hormones were significantly

Table 3. Age-Adjusted Regression Coefficients between Vascular Measurements and Plasma Hormones

	%FMD	Carotid IMT
Estradiol	0.023	-0.098
Testosterone	0.029	-0.117
DHEA-S	0.366†	-0.146
Cortisol	0.156	-0.062

%FMD, percent flow-mediated dilation of brachial artery; IMT, intima-media thickness of common carotid artery. Standardized regression coefficients by multiple regression analyses with %FMD or carotid IMT as a dependent variable and age and each of the hormones as independent variables are shown. † $p < 0.01$.

changed according to coronary risk factors or medications (data not shown).

Relationship between Plasma Hormones and Vascular Measurements

First, simple correlation coefficients between plasma hormones and vascular measurements were determined. As shown in Table 2, %FMD was positively correlated with DHEA-S, whereas carotid IMT was negatively correlated with DHEA-S. There was no significant correlation between vascular measurements and other steroid hormones.

Next, age-adjusted regression coefficients were determined, because age was correlated with some of the hormones and vascular measurements, as mentioned above. The results showed that none of the hormones were significantly related to carotid IMT (Table 3). In contrast, DHEA-S was significantly related to %FMD, independent of age. Furthermore, the age-adjusted regression coefficients between DHEA-S and %FMD were 0.374 in the subjects with no coronary risk factor or medication ($p < 0.05$, $n = 31$) and 0.399 in those with coronary risk factors ($p < 0.05$, $n = 84$), most of whom were taking some medications.

Finally, multiple regression analyses were performed to exclude the influence of coronary risk factors on the relationship between hormones and %FMD. As shown in Table 4, DHEA-S was related to %FMD, independent of age, body mass index, hypertension, hyperlipidemia, diabetes mellitus and current smoking (Model 1), and were independent of age, body mass index, systolic blood pressure, total cholesterol, high-density lipoprotein (HDL) cholesterol, fasting plasma glucose and current smoking (Model 2). Furthermore, the relationship between DHEA-S and %FMD was significant after addition of carotid IMT to the model (Model 3), suggesting that the relationship was not attributable to the effect of DHEA(-S) on the development of subclinical atherosclerosis. Also, the statistical result was unchanged after addition of nitroglycerin-induced dilation of the brachial artery to the model (Model 4), indicating that DHEA-S is related to endothelial function independent of arterial compliance. Estradiol,

Table 4. Regression Coefficients between %FMD and Plasma Hormones Adjusted for Coronary Risk Factors

	Model 1	Model 2	Model 3	Model 4
Estradiol	0.065	0.042	0.025	0.094
Testosterone	0.036	0.052	0.028	0.148
DHEA-S	0.344 [†]	0.291*	0.263*	0.250*
Cortisol	0.150	0.127	0.099	0.110

Standardized regression coefficients by multiple regression analyses with %FMD as a dependent variable and coronary risk factors (covariates used in each analysis are listed below) and each of the hormones as independent variables are shown. [†] $p < 0.01$, * $p < 0.05$. Model 1: age, body mass index, hypertension, hyperlipidemia, diabetes mellitus, and current smoking. Model 2: age, body mass index, systolic blood pressure, total cholesterol, high-density lipoprotein cholesterol, fasting plasma glucose and current smoking. Model 3: Model 2 plus carotid intima-media thickness. Model 4: Model 2 plus percent nitroglycerin-induced dilation of brachial artery. %FMD, percent flow-mediated dilation of brachial artery.

testosterone and cortisol were not significantly related to %FMD in similar multivariate analyses (Table 4). As shown in Fig. 1, %FMD showed a stepwise increment according to quartiles of DHEA-S, and %FMD in the highest quartile of free testosterone was 1.8-fold higher than that in the lowest quartile (5.3 ± 1.3 vs. 2.9 ± 2.0 , $p < 0.01$).

All the variables except hormones were mandatorily incorporated into the multiple regression models shown in Table 4, because they are classical coronary risk factors. In forward and backward stepwise models including age, body mass index, hypertension, hyperlipidemia, diabetes mellitus, smoking and the four steroid hormones, DHEA-S, hypertension and hyperlipidemia ($\beta = 0.390$, -0.224 and -0.295 , respectively) were selected as significant variables. Next, in forward and backward stepwise models including age, body mass index, systolic blood pressure, total cholesterol, HDL cholesterol, fasting plasma glucose, smoking and the four steroid hormones, only DHEA-S and glucose were selected as significant variables ($\beta = 0.330$ and -0.325 , respectively). Taken together, these results show that DHEA-S was consistently related to %FMD in the multivariate analyses.

Discussion

In this cross-sectional study, plasma DHEA-S levels were positively correlated with %FMD, a surrogate marker of clinical atherosclerosis that reflects endothelial function (18, 19). Adjustment for potential confounders such as age, coronary risk factors and nitroglycerin-induced dilation of the brachial artery had little influence on the results. These results suggest that endogenous DHEA(-S) plays a vasoprotective role in postmenopausal women.

The majority of DHEA exists as the sulfated form (DHEA-S) in circulation, and its concentration is reported to be 100-

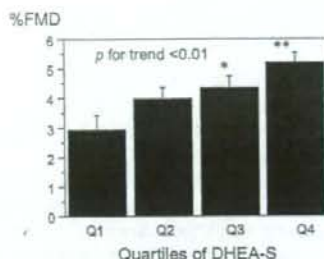


Fig. 1. Percent flow-mediated dilation of the brachial artery (%FMD) according to quartiles of serum dehydroepiandrosterone-sulfate (DHEA-S). Values are expressed as the means \pm SEM. * $p < 0.05$, ** $p < 0.01$ vs. Q1.

to 500-fold higher than that of testosterone and 1,000- to 10,000-fold higher than that of estradiol (4), suggesting an important physiologic role of DHEA(-S). Plasma DHEA-S levels do not drop during the menopause transition, but decline with aging (1, 2, 20). A recent study has shown a positive correlation between DHEA-S levels and the duration of reactive hyperemia, another less specific marker of endothelial function compared to FMD, in young women (26 \pm 6 years old, $n = 27$) with polycystic ovary syndrome (21). In postmenopausal women, however, a correlation between DHEA-S levels and endothelial vasomotor function has not yet been reported. On the other hand, two studies have reported the effects of DHEA supplementation on FMD in postmenopausal women (12, 22). Williams *et al.* (12) showed a significant increase in FMD after the 12-week administration of DHEA (50 mg/day) in healthy postmenopausal women, while Silvestri *et al.* (22) observed no effect using the same dose of DHEA for 4 weeks in postmenopausal women with increased cardiovascular risk. In middle-aged men, it has been reported that low-dose DHEA supplementation (25 mg/day) for 12 weeks improved FMD (23). Experimental studies have also demonstrated the effects of DHEA on endothelial proliferation (12) and endothelial nitric oxide synthase (24), further supporting the notion that DHEA(-S) has a protective effect on the endothelium.

At this moment, intrinsic receptors for DHEA(-S) have not been identified. Thus, DHEA(-S) may exert its activity after conversion to androgens or estrogens via androgen or estrogen receptors, although in previous reports neither an androgen receptor antagonist nor an estrogen receptor antagonist blocked some of the *in vitro* effects of DHEA on endothelial cells (12, 24). Testosterone supplementation in men (25, 26) and estrogen replacement in postmenopausal women (27, 28) improves endothelial vasomotor function. Activation of endothelial nitric oxide synthase and stimulation of nitric oxide production via androgen and estrogen receptors (29) might be attributable to the effect of DHEA(-S) on FMD. For

this reason, we added testosterone and estradiol into the analysis. Plasma testosterone and estradiol levels, however, were correlated neither with %FMD nor with DHEA-S (data not shown), although tissue conversion of DHEA(-S) into androgens or estrogens might play a role. Further studies are needed to clarify the molecular mechanism underlying the association between DHEA-S and FMD.

The incidence of cardiovascular disease is lower in premenopausal women than in men of the same age, and increases after menopause (30), indicating the cardioprotective effect of endogenous estrogen in premenopausal women. Accordingly, estrogen replacement therapy had been expected to prevent cardiovascular disease in postmenopausal women, with disappointing results in randomized controlled trials (31). Alternatively, there has been a resurgence of interest in DHEA as an anti-aging hormone, and DHEA has been widely used in this context in the USA despite the dearth of information on its physiologic and pharmacologic effects (4). Although the present study indicated a cardioprotective effect of endogenous DHEA(-S), we do not suggest that DHEA be prescribed for postmenopausal women with coronary risk factors until the efficacy and safety of DHEA supplementation has been established. At present, life-style modification such as exercise is preferable, because exercise can increase both DHEA levels (32) and nitric oxide production (33) in elderly women.

The results of this study do not imply that DHEA(-S) has favorable effects on endothelial function in men. In fact, the plasma DHEA-S level was not related to FMD in middle-aged men after adjustments for age and coronary risk factors (our unpublished observation). Consequently, the meaning of DHEA-S in association with endothelial function may be different between men and women. Gender differences in other steroid hormones and steroid hormone receptor expression in arteries (34, 35) might play a mechanistic role.

This study had some limitations. First, since this was a cross-sectional study, the causal relationship between DHEA-S and vasomotor function could not be determined. Endothelial dysfunction might be associated with a reduction in blood flow of the adrenal glands, leading to decreased hormone production. Longitudinal studies following the subjects might add some information in this regard. Secondly, a population bias was possible. Three-quarters of the study subjects had one or more of the coronary risk factors, and most of them were taking medications. Consequently, the results might have been different if subjects homogeneous in terms of health status and medications had been studied, although the association of DHEA-S with %FMD was consistent in the subjects with or without coronary risk factors and medications, as mentioned above. The subjects with a history of cardiovascular disease were excluded from the study, because they might have low plasma DHEA-S levels as a result of advanced atherosclerosis and reduced blood flow of the adrenal glands, although the inclusion of those subjects did not fundamentally alter the statistical results (data not shown).

In summary, low plasma DHEA-S levels were associated with endothelial dysfunction in postmenopausal women independent of other risk factors, suggesting a protective effect of DHEA(-S) on the endothelium. This finding provides a mechanistic insight into the role of endogenous DHEA in the development of cardiovascular disease in postmenopausal women.

Acknowledgements

We thank Ms. Yuki Ito for her excellent technical assistance.

References

1. Bjornerem A, Straume B, Midtby M, et al: Endogenous sex hormones in relation to age, sex, lifestyle factors, and chronic diseases in a general population: the Tromso Study. *J Clin Endocrinol Metab* 2004; **89**: 6039-6047.
2. Tannenbaum C, Barrett-Connor E, Laughlin GA, Platt RW: A longitudinal study of dehydroepiandrosterone sulphate (DHEAS) change in older men and women: the Rancho Bernardo Study. *Eur J Endocrinol* 2004; **151**: 717-725.
3. Lamberts SW, van den Beld AW, van der Lely AJ: The endocrinology of aging. *Science* 1997; **278**: 419-424.
4. Hinson JP, Raven PW: DHEA deficiency syndrome: a new term for old age? *J Endocrinol* 1999; **163**: 1-5.
5. Wu FC, von Eckardstein A: Androgens and coronary artery disease. *Endocr Rev* 2003; **24**: 183-217.
6. Liu PY, Death AK, Handelsman DJ: Androgens and cardiovascular disease. *Endocr Rev* 2003; **24**: 313-340.
7. Tchernof A, Labrie F: Dehydroepiandrosterone, obesity and cardiovascular disease risk: a review of human studies. *Eur J Endocrinol* 2004; **151**: 1-14.
8. Haffner SM, Moss SE, Klein BE, Klein R: Sex hormones and DHEA-SO₄ in relation to ischemic heart disease mortality in diabetic subjects. The Wisconsin Epidemiologic Study of Diabetic Retinopathy. *Diabetes Care* 1996; **19**: 1045-1050.
9. Bernini GP, Sgro' M, Moretti A, et al: Endogenous androgens and carotid intimal-medial thickness in women. *J Clin Endocrinol Metab* 1999; **84**: 2008-2012.
10. Santoro N, Torrens J, Crawford S, et al: Correlates of circulating androgens in mid-life women: the study of women's health across the nation. *J Clin Endocrinol Metab* 2005; **90**: 4836-4845.
11. Barna I, Feher T, de Chatel R: Relationship between blood pressure variability and serum dehydroepiandrosterone sulfate levels. *Am J Hypertens* 1998; **11**: 532-538.
12. Williams MR, Dawood T, Ling S, et al: Dehydroepiandrosterone increases endothelial cell proliferation *in vitro* and improves endothelial function *in vivo* by mechanisms independent of androgen and estrogen receptors. *J Clin Endocrinol Metab* 2004; **89**: 4708-4715.
13. Hutchison SJ, Browne AE, Ko E, et al: Dehydroepiandrosterone sulfate induces acute vasodilation of porcine coronary arteries *in vitro* and *in vivo*. *J Cardiovasc Pharmacol* 2005; **46**: 325-332.
14. Japanese Society of Hypertension: Japanese Society of Hypertension Guidelines for the Management of Hyperten-

- sion (JSH 2004). *Hypertens Res* 2006; **29** (Suppl): S1-S105.
15. Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults: Executive Summary of the Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). *JAMA* 2001; **285**: 2486-2497.
 16. American Diabetes Association: Screening for diabetes (position statement). *Diabetes Care* 2001; **24** (Suppl): S21-S24.
 17. Hashimoto M, Eto M, Akishita M, et al: Correlation between flow-mediated vasodilatation of the brachial artery and intima-media thickness in the carotid artery in men. *Arterioscler Thromb Vasc Biol* 1999; **19**: 2795-2800.
 18. Tarutani Y, Matsumoto T, Takashima H, Yamane T, Horie M: Brachial artery flow-mediated vasodilation is correlated with coronary vasomotor and fibrinolytic responses induced by bradykinin. *Hypertens Res* 2005; **28**: 59-66.
 19. Raitakari OT, Celermajer DS: Flow-mediated dilatation. *Br J Clin Pharmacol* 2000; **50**: 397-404.
 20. Randolph JF Jr, Sowers M, Gold EB, et al: Reproductive hormones in the early menopausal transition: relationship to ethnicity, body size, and menopausal status. *J Clin Endocrinol Metab* 2003; **88**: 1516-1522.
 21. Dagne A, Lekakis J, Mihos C, et al: Association of dehydroepiandrosterone-sulfate with endothelial function in young women with polycystic ovary syndrome. *Eur J Endocrinol* 2006; **154**: 883-890.
 22. Silvestri A, Gambacciani M, Vitale C, et al: Different effect of hormone replacement therapy, DHEAS and tibolone on endothelial function in postmenopausal women with increased cardiovascular risk. *Maturitas* 2005; **50**: 305-311.
 23. Kawano H, Yasue H, Kitagawa A, et al: Dehydroepiandrosterone supplementation improves endothelial function and insulin sensitivity in men. *J Clin Endocrinol Metab* 2003; **88**: 3190-3195.
 24. Simoncini T, Mannella P, Fornari L, Varone G, Caruso A, Genazzani AR: Dehydroepiandrosterone modulates endothelial nitric oxide synthesis via direct genomic and nongenomic mechanisms. *Endocrinology* 2003; **144**: 3449-3455.
 25. Ong PJ, Patrizi G, Chong WC, Webb CM, Hayward CS, Collins P: Testosterone enhances flow-mediated brachial artery reactivity in men with coronary artery disease. *Am J Cardiol* 2000; **85**: 269-272.
 26. Kang SM, Jang Y, Kim JY, et al: Effect of oral administration of testosterone on brachial arterial vasoreactivity in men with coronary artery disease. *Am J Cardiol* 2002; **89**: 862-864.
 27. Wakatsuki A, Okatani Y, Ikenoue N, Fukaya T: Different effects of oral conjugated equine estrogen and transdermal estrogen replacement therapy on size and oxidative susceptibility of low-density lipoprotein particles in postmenopausal women. *Circulation* 2002; **106**: 1771-1776.
 28. Hashimoto M, Miyao M, Akishita M, et al: Effects of long-term and reduced-dose hormone replacement therapy on endothelial function and intima-media thickness in postmenopausal women. *Menopause* 2002; **9**: 58-64.
 29. Khalil RA: Sex hormones as potential modulators of vascular function in hypertension. *Hypertension* 2005; **46**: 249-254.
 30. Kannel WB, Hjortland MC, McNamara PM, Gordon T: Menopause and risk of cardiovascular disease: the Framingham Study. *Ann Intern Med* 1976; **85**: 447-452.
 31. Rossouw JE, Anderson GL, Prentice RL, et al: Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results from the women's health initiative randomized controlled trial. *JAMA* 2002; **288**: 321-333.
 32. Akishita M, Yamada S, Nishiya H, Sonohara K, Nakai R, Toba K: Effects of physical exercise on plasma concentrations of sex hormones in elderly women with dementia. *J Am Geriatr Soc* 2005; **53**: 1076-1077.
 33. Maeda S, Tanabe T, Otsuki T, et al: Moderate regular exercise increases basal production of nitric oxide in elderly women. *Hypertens Res* 2004; **27**: 947-953.
 34. Liu PY, Christian RC, Ruan M, Miller VM, Fitzpatrick LA: Correlating androgen and estrogen steroid receptor expression with coronary calcification and atherosclerosis in men without known coronary artery disease. *J Clin Endocrinol Metab* 2005; **90**: 1041-1046.
 35. Christian RC, Liu PY, Harrington S, Ruan M, Miller VM, Fitzpatrick LA: Intimal estrogen receptor (ER)beta, but not ERalpha expression, is correlated with coronary calcification and atherosclerosis in pre- and postmenopausal women. *J Clin Endocrinol Metab* 2006; **91**: 2713-2720.

REFERENCES

1. Ellison DH, Berl T. Clinical practice. The syndrome of inappropriate antidiuresis. *N Engl J Med* 2007;356:2064-2072.
2. Robertson GL. Regulation of arginine vasopressin in the syndrome of inappropriate antidiuresis. *Am J Med* 2006;119:S36-S42.
3. McKeith IG, Dickson DW, Lowe J et al. Diagnosis and management of dementia with Lewy bodies: Third report of the DLB Consortium. *Neurology* 2005; 65:1863-1872.
4. Walker MP, Ayre GA, Cummings JL et al. The Clinician Assessment of Fluctuation and the One Day Fluctuation Assessment Scale. Two methods to assess fluctuating confusion in dementia. *Br J Psychiatry* 2000;177:252-256.
5. Sone H, Okuda Y, Bannai C et al. Syndrome of inappropriate secretion of antidiuretic hormone (SIADH) and Gerhardt syndrome associated with Shy-Drager syndrome. *Intern Med* 1994;33:773-778.
6. Bridges TE, Hillhouse EW, Jones MT. The effect of dopamine on neurohypophysial hormone release in vivo and from the rat neural lobe and hypothalamus in vitro. *J Physiol* 1976;260:647-666.
7. Renneboog B, Musch W, Vandemergel X et al. Mild chronic hyponatremia is associated with falls, unsteadiness, and attention deficits. *Am J Med* 2006; 119:e1-e8.

INCREASE IN OXIDATIVE STRESS LEVELS IN ELDERLY PATIENTS WITH OBSTRUCTIVE SLEEP APNEA SYNDROME: EFFECTS OF AGE AND SEX

To the Editor: Obstructive sleep apnea syndrome (OSAS) has emerged as an important risk factor for cardiovascular disease.¹⁻³ Although sleep apnea increases with age, the pathological role of OSAS has not been completely established in elderly people. OSAS-induced hypoxic and oxidative stress have been implicated in the increase in circulating inflammatory mediators, including adhesion molecules, inflammatory cytokines, and high-sensitivity C-reactive protein, leading to hypertension and cardiovascular events.⁴ The fluctuations in the level of oxygen saturation can be considered analogous to recurrent episodes of ischemia-reperfusion injury, which causes damage after the restoration of blood flow to ischemic or hypoxic tissues. In patients with untreated OSAS, an increase in the production of reactive oxygen species (ROS) and plasma lipid peroxides and a reduced antioxidant capacity have been demonstrated.^{5,6} Although oxidative stress increases with age,⁷ the effects of age and sex on the oxidative stress in OSAS patients have not been elucidated. It was hypothesized that the increase in age and the severity of OSAS might be correlated with oxidative stress. In the current study, oxidative stress was assessed based on urinary levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG), a marker of deoxyribonucleic acid (DNA) oxidation in vivo.

Age-dependent differences in urinary levels of 8-OHdG were examined in patients with OSAS. The experimental group consisted of 80 middle-aged (<60) and 80 elderly (≥ 60) patients with OSAS; their age and body mass index (BMI) matched those of the individuals in the control group comprising 80 middle-aged and 80 elderly subjects without OSAS. The patients had to fulfill the following criteria: no renal or renovascular hypertension, systolic blood pressure (BP) less than 160 mmHg or diastolic BP less than 95 mmHg, no chronic renal or hepatic disease, and no diabetes mellitus. Patients with a history of smoking or systemic infections at the time of the study or in the 4 weeks before the study were excluded. No patients were treated

using antihypertensive agents. The subjects were examined using polysomnography (PSG) and classified as control subjects based on the apnea-hypopnea index (AHI). In this study, to assess OSAS-induced hypoxia quantitatively, the oxyhemoglobin desaturation index (ODI) was used as previously described (90%).⁴ ODI was defined as $DI = \Sigma(90 - SaO_2) t$, where t represents the time of desaturation. The urinary excretion levels of 8-OHdG and sleep study variables were compared. Moreover, the correlations between various parameters of OSAS, including the ODI, and oxidative stress in middle-aged and elderly patients with OSAS, were evaluated. All urine and serum samples were collected in the morning between 7:30 and 8:00 a.m. after the PSG examination. The 8-OHdG concentration was determined using an enzyme-linked immunosorbent assay (ELISA) kit (Japan Institute for the Control of Aging; Nikken SEIL Corporation, Fukuroi, Shizuoka, Japan), and urinary creatinine concentration was determined using a standard automated colorimetric assay. Thereafter, urinary 8-OHdG level was normalized for urinary creatinine level and is presented as urinary 8-OHdG (ng/mL):creatinine (mg/mL) ratio. A stable correlation between spot urine levels and 24-hour excretion levels of 8-OHdG has already been established (Table 1).⁸

There were no significant differences in BMI between patients and control subjects in each age group, although AHI in the OSAS group was markedly higher than in the control group. There were no significant differences in BP or metabolic indices. The AHI values in the elderly (50.1 ± 3.2 events/h) and middle-aged (51.6 ± 3.0 events/h) patients with OSAS were considerably greater than those for the age-matched controls (3.8 ± 0.3 and 3.6 ± 0.4 events/h, respectively). There were significant differences in baseline ODI values between the OSAS patients and controls, suggesting that the OSAS patients were exposed to a significantly greater degree of hypoxia than the control subjects. There were no differences in ODI value between the middle-aged and elderly OSAS patients.

The uncorrected values of 8-OHdG levels in urine partially collected during the early morning hours in middle-aged and elderly patients with OSAS were significantly greater than those in the age- and BMI-matched controls. The creatinine-corrected values in all the patients with OSAS were also greater than those in the controls, although age did not affect the urinary excretion levels of 8-OHdG in patients with OSAS. In contrast, the creatinine-corrected 8-OHdG values in elderly women (5.6 ± 1.1 ng/mL) were greater than those in middle-aged women (4.4 ± 0.9 ng/mL) in the control group. This age-dependent difference in 8-OHdG levels was not observed in the female patients with OSAS, and no age-dependent differences in 8-OHdG levels were observed between male controls and male patients.

A positive relationship was noted between 8-OHdG levels and AHI or the magnitude of arterial oxygen desaturation, as indicated by ODI. This significant correlation is observed more clearly between 8-OHdG levels and hypoxic episodes (ODI) (correlation coefficient (r) = 0.389, $P < .01$) than between 8-OHdG levels and apnea episodes (AHI) ($r = 0.249$, $P < .05$).

These results indicate that oxidative stress, as indicated by urinary 8-OHdG excretion level, increased with age in obese females without sleep apnea but was not observed in

Table 1. Urinary 8-Hydroxy-2'-Deoxyguanosine (8-OHdG) Levels and Other Variables in Elderly and Middle-Aged Subjects with Obstructive Sleep Apnea Syndrome (OSAS) and Controls

Variable	Middle-Aged Group with OSAS (n = 80)	Elderly Group with OSAS (n = 80)	Middle-Aged Control Group (n = 80)	Elderly Control Group (n = 80)
	Mean ± Standard Error			
Age	46.8 ± 2.2	65.8 ± 2.2	45.1 ± 2.2	64.8 ± 2.1
Body mass index, kg/m ²	33.4 ± 0.9	32.1 ± 0.9	32.8 ± 1.1	31.5 ± 1.1
Systolic blood pressure, mmHg	138.1 ± 3.7	138.1 ± 4.7	135.6 ± 4.1	137.1 ± 3.7
Diastolic blood pressure, mmHg	82.1 ± 3.2	80.1 ± 3.2	78.9 ± 3.8	78.1 ± 2.8
Total cholesterol, mg/dL	202.9 ± 7.9	199.1 ± 6.9	202.9 ± 7.9	198.3 ± 10.8
High-density lipoprotein cholesterol, mg/dL	43.0 ± 2.2	41.0 ± 2.1	43.6 ± 2.3	42.2 ± 2.1
Triglyceride, mg/dL	144.1 ± 10.7	137.1 ± 12.7	140.1 ± 10.7	132.0 ± 10.9
Fasting plasma glucose, mg/dL	98.4 ± 1.3	94.6 ± 1.3	97.6 ± 1.4	90.3 ± 1.1
Hemoglobin A1c, %	5.7 ± 0.1	5.8 ± 0.1	5.6 ± 0.1	5.27 ± 0.1
Total sleep time, minutes	368.1 ± 20.3*	348.1 ± 20.3*	440.3 ± 20.9	414.3 ± 20.9
Apnea-hypopnea index, events/h	51.6 ± 3.0*	50.1 ± 3.2*	3.8 ± 0.3	3.6 ± 0.4
Lowest oxygen saturation, %	67.2 ± 2.1*	68.9 ± 3.0*	95.8 ± 0.5	94.1 ± 0.5
Oxyhemoglobin desaturation index	2.45 ± 0.32*	2.41 ± 0.36*	0.02 ± 0.01	0.02 ± 0.01
Arousal index/h	42.4 ± 3.1*	40.2 ± 2.2*	8.3 ± 3.1	8.3 ± 3.1
8-OHdG, ng/mL	16.3 ± 3.7	17.1 ± 4.1*	7.8 ± 1.9	8.9 ± 2.1
8-OHdG/Cr, ng/mg	9.7 ± 1.9	10.1 ± 2.1*	4.7 ± 0.9	5.3 ± 1.1

There were 40 men and 40 women in each group.

*P < .001 versus control group.

8-OHdG/Cr = creatinine-corrected 8-OHdG.

obese males without sleep apnea. Furthermore, greater DNA oxidation in elderly patients with OSAS was observed to a considerable extent and in a similar manner, than in middle-aged patients with OSAS, irrespective of sex. This is because urinary 8-OHdG excretion levels were significantly correlated with severity of hypoxia as indexed according to ODI. The greater oxidative stress resulted from the considerable hypoxic stress rather than the apnea episode itself.⁹ Age-dependent differences in 8-OHdG levels were observed in the female control subjects but not in female patients with OSAS. Thus, the augmented increase in 8-OHdG secretion caused by severe nocturnal hypoxemia and oxidative stress due to OSAS may overcome the age-dependent increase in 8-OHdG levels in elderly patients with OSAS.

Because life-threatening diseases such as arteriosclerosis and cancer and senescence may be induced and progress as a result of oxidative stress due to ROS, the reduction of oxidative stress is important to prevent the incidence of these events. Although the pathological role of sleep apnea in cardiovascular events and mortality in elderly patients with OSAS remains controversial, the current study indicates that elderly patients are exposed to significant oxidative stress due to sleep apnea-related nocturnal hypoxemia.

Treatment with continuous positive airway pressure (CPAP) greatly reduces hypoxic stress in patients with OSAS. Thus, the treatment of OSAS with CPAP may be clinically effective for the prevention of cardiovascular complications even in elderly OSAS patients, irrespective of sex.

Shinji Teramoto, MD
Yasuhiro Yamaguchi, MD
Hiroshi Yamamoto, MD

Yoko Hanaoka, MD
Masaki Ishii, MD
Hibi Shinichiro, MD
Haruki Kume, MD
Masahiro Akishita, MD
Yasuyoshi Ouchi, MD
Department of Geriatric Medicine
and Urology Graduate School of Medicine
The University of Tokyo
Tokyo, Japan

ACKNOWLEDGMENTS

Conflict of Interest: This work was supported in part by a research grant from Mitsui Life Social Welfare Foundation Japan fund and by a research grant from Mitsukoshi Health and Welfare Foundation in Japan. There is no conflict of interest regarding the present study.

Author Contributions: Study concept and design: Shinji Teramoto and Yasuhiro Yamaguchi. Acquisition of subjects and data, analysis: Hiroshi Yamamoto, Yoko Hanaoka, Masaki Ishii, Hibi Shinichiro. Interpretation of data: Haruki Kume and Masahiro Akishita. Preparation of manuscript: Yasuyoshi Ouchi.

Sponsor's Role: No sponsor in this study.

REFERENCES

- Chhajed PN, Tamm M, Strobel W. Sleep apnea and heart disease. *N Engl J Med* 2006;354:1086-1089.
- Teramoto S, Kume H, Matsuse T. Ambulatory blood pressure after sleep apnoea treatment. *Lancet* 2002;360:341-342.
- Teramoto S, Ohga E, Ouchi Y. Obstructive sleep apnoea. *Lancet* 1999;354:1213-1214.

4. Teramoto S, Yamamoto H, Ouchi Y. Increased C-reactive protein and increased plasma interleukin-6 may synergistically affect the progression of coronary atherosclerosis in obstructive sleep apnea syndrome. *Circulation* 2003;107:E40.
5. Dyugovskaya I, Lavie P, Lavie L. Increased adhesion molecules expression and production of reactive oxygen species in leukocytes of sleep apnea patients. *Am J Respir Crit Care Med* 2002;165:934-939.
6. Barcelo A, Miralles C, Barbe F et al. Abnormal lipid peroxidation in patients with sleep apnoea. *Eur Respir J* 2000;16:644-647.
7. Linnane AW, Marzuki S, Ozawa T et al. Mitochondrial DNA mutations as an important contributor to ageing and degenerative diseases. *Lancet* 1989;1:642-645.
8. Nakano M, Kawanishi Y, Kamohara S et al. Oxidative DNA damage (8-hydroxydeoxyguanosine) and body iron status: A study on 2507 healthy people. *Free Radic Biol Med* 2003;35:826-832.
9. Yamauchi M, Nakano H, Maekawa J et al. Oxidative stress in obstructive sleep apnea. *Chest* 2005;127:1674-1679.

DIAGNOSTIC ACCURACY OF CRITERIA FOR URINARY TRACT INFECTION IN NURSING HOMES

To the Editor: The article by Juthani-Mehta et al.¹ illustrates the dilemma facing clinicians taking care of residents of long-term care facilities (LTCFs). As the authors proposed, "a different combination of existing clinical criteria and geriatric manifestations will be more accurate." The challenge is how to differentiate the clinical manifestations of urinary tract infection (UTI) from coexisting comorbidities in older adults.

One study of 284 geriatric patients with UTI in the emergency department considered the following symptoms as possible manifestations of UTI: abdominal pain, nausea, vomiting, decreased appetite, dizziness, malaise, weakness, confusion, falls, and mental status changes.² Most of the symptoms, if not all, are based on clinical observation and have not been validated by clinical research studies. In addition, these symptoms are clearly nonspecific, although findings from other studies suggest an indirect link between episodes of falls and UTI, because older adults with UTI may experience delirium, which can lead to falls and fractures. A prospective study of 199 patients in five residential care facilities during 1 year of follow-up revealed that delirium and acute UTI were considered major factors precipitating falls.³ A case-control study of 335 residents living in an LTCF revealed that altered mental state was recognized as the most important risk factor for injury in those who fell,⁴ although a direct link between falls and UTI has not been demonstrated.

The effect of UTI on the functional capacity (e.g., oral intake, activities of daily living) of residents of LTCFs is not clear. A prospective study of 1,324 residents in 39 nursing homes in western Switzerland examined the relationship between infections and functional impairment (defined as death or a decreased activity of daily living score at the end of each follow-up period) in residents of LTCFs during a 6-month follow-up period.⁵ This study revealed that infection appeared to be a cause and a consequence of functional impairment in nursing home residents, although subgroup analyses based on the type of infection revealed no significant increase in the risk of functional impairment for UTI. A 3-month period between functional assessments in this study may not have been sufficiently sensitive to detect transient changes in functional status during an episode of UTI.

Because of their nonspecific nature, apart from local urinary tract symptoms and fever, geriatric manifestations of UTI in elderly people in LTCFs may not be sufficient to differentiate UTIs from other coexisting disorders. Presently, the individual healthcare provider must make the final judgment as to when to order urinalysis and whether a patient with bacteriuria has a UTI and should therefore receive antibiotics.⁶ More evidence-based studies are needed to clarify this dilemma facing clinicians in the care of elderly people in the long-term care setting.

Jen-Tzer Gau, MD

Department of Geriatric Medicine and Gerontology

Steven Clay, DO

Department of Family Medicine

College of Osteopathic Medicine

Ohio University

Athens, OH

ACKNOWLEDGMENTS

Conflict of Interest: The editor in chief has reviewed the conflict of interest checklists provided by the author and has determined that none of the authors have any financial or any other kind of personal conflicts with this letter.

Author Contributions: Dr. Gau is responsible for the letter. Dr. Clay contributed to the editing and preparation of the letter.

Sponsor's Role: None.

REFERENCES

1. Juthani-Mehta M, Tinetti M, Perrelli E et al. Diagnostic accuracy of criteria for urinary tract infection in a cohort of nursing home residents. *J Am Geriatr Soc* 2007;55:1072-1077.
2. Ginde AA, Rhee SH, Katz ED. Predictors of outcome in geriatric patients with urinary tract infections. *J Emerg Med* 2004;27:101-108.
3. Kallin K, Jensen J, Olsson LL et al. Why the elderly fall in residential care facilities, and suggested remedies. *J Fam Pract* 2004;53:41-52.
4. Krueger PD, Brazil K, Lohfeld LH. Risk factors for falls and injuries in a long-term care facility in Ontario. *Can J Public Health* 2001;92:117-120.
5. Bels CJ, Ghilardi G, Wietlisbach V et al. Infections and functional impairment in nursing home residents: A reciprocal relationship. *J Am Geriatr Soc* 2004;52:700-706.
6. Johnson JR. Laboratory diagnosis of urinary tract infections in adult patients. *Clin Infect Dis* 2004;39:873.

THE AGING POPULATION AND DEVELOPMENT OF GERIATRICS IN CHINA

To the Editor: We read with great interest the article by Flaherty et al.,¹ in which they provided an overview of the population demographics and healthcare statistics in the People's Republic of China focusing on the older Chinese population, its current care system, and geriatrics. We appreciate the authors and the Journal for the interest in introducing the largest aging population in world to the American geriatrics community. In addition to congratulating the authors' accomplishment of such an overview, we have the following comments.



Raloxifene analogue LY117018 suppresses oxidative stress-induced endothelial cell apoptosis through activation of ERK1/2 signaling pathway

Jing Yu ^{a,b}, Masato Eto ^a, Koichi Kozaki ^a, Masahiro Akishita ^a, Tetsuro Okabe ^b, Yasuyoshi Ouchi ^{a,*}

^a Department of Geriatric Medicine, Graduate School of Medicine, University of Tokyo, Tokyo, Japan

^b Department of Integrated Traditional Medicine, Graduate School of Medicine, University of Tokyo, Tokyo, Japan

ARTICLE INFO

Article history:

Received 29 June 2007

Received in revised form 11 April 2008

Accepted 23 April 2008

Available online 5 May 2008

Keywords:

Endothelial cell

Apoptosis

Selective estrogen receptor modulator

Raloxifene

ERK1/2

ABSTRACT

A selective estrogen receptor modulator, raloxifene, has been shown to reduce cardiovascular events in relatively high-risk postmenopausal women with osteoporosis. However, the mechanisms by which raloxifene exerts a pharmacological effect on cardiovascular organs have not been fully elucidated. The present study was designed to examine whether the raloxifene analogue, 6-hydroxy-2-(*p*-hydroxyphenyl)-benzo(b) thien-3-yl-*p*-(2-(pyrrolidinyl)ethoxy phenyl ketone (LY117018), could inhibit apoptosis and to clarify the signaling pathway in vascular endothelial cells. LY117018 significantly inhibited hydrogen peroxide-induced apoptosis in bovine carotid artery endothelial cells. The anti-apoptotic effect of LY117018 was abolished by an estrogen receptor antagonist, 7 α ,7 β -[9]([4,4,5,5,5-Pentafluoropentyl)sulfonyl]nonyl]estra-1,3,5(10)-triene-3,17-diol (ICI 162,780). Mitogen-activated protein kinases (MAPK), including p38, c-Jun N-terminal kinase (JNK) and extracellular signal-regulated protein kinase1/2 (ERK1/2), and Akt, have been shown to act as apoptotic or anti-apoptotic signals. Phosphorylation of p38, JNK, ERK1/2 and Akt was examined. LY117018 increased ERK1/2 phosphorylation but did not enhance the phosphorylation of p38, JNK, or Akt. The anti-apoptotic effect of LY117018 was prevented by treatment with 2-[2'-amino-3'-methoxyphenyl]-oxanaphthalen-4-one (PD98059), an upstream inhibitor of ERK1/2. LY117018 stimulated an increase in ERK1/2 phosphorylation, which was diminished by ICI 162,780. The activation of ERK1/2 by LY117018 was not inhibited by the transcription inhibitor, actinomycin D. These results suggest that estrogen receptors and the ERK1/2 signaling pathway are involved in the anti-apoptotic action of LY117018 in vascular endothelial cells.

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1. Introduction

The incidence of clinical coronary heart disease in premenopausal women is very low. However, following the menopause, atherogenic risk factors increase and the rate of clinical coronary events accelerates to the level observed in men (Kannel et al., 1976). This difference has been considered to be attributable to the protective effects of estrogen before the menopause (Clarkson, 2007). Recent randomized placebo-controlled trials of hormone replacement therapy, however, have not shown any benefit in either the secondary or the primary prevention of cardiovascular events (Hulley et al., 1998; Grady et al., 2002; Rossouw et al., 2002).

Much current interest is focused on the therapeutic potential of selective estrogen receptor modulators. Interestingly, drugs of this class show estrogen-antagonist effects in the mammary gland and uterus, while they have estrogen-agonist effects in bone and other

tissues (Delmas et al., 1997; Grady et al., 2004; Johnell et al., 2004; Cox et al., 2004; Sporn et al., 2004). Thus, they are expected to overcome the adverse effects found with conventional hormone replacement therapy.

Recently, the MORE (Multiple Outcomes of Raloxifene Evaluation) study showed that a representative selective estrogen receptor modulator, raloxifene, significantly reduced cardiovascular events in relatively high-risk postmenopausal women with osteoporosis (Barrett-Connor et al., 2002). The death of endothelial and vascular smooth muscle cells is implicated in several pathological vascular conditions, such as atherosclerosis and aneurysm formation. Endothelial damage/dysfunction plays a central role in the clinical manifestation of coronary atherosclerosis (Ross, 1990; Ross, 1999). It has been reported that selective estrogen receptor modulators show a variety of direct actions on vascular cells via estrogen receptors (Simoncini et al., 1999; Simoncini et al., 2002). However, the effect of selective estrogen receptor modulators on endothelial apoptosis has not been clarified.

The aim of this study was to examine the effect of a raloxifene analogue, 6-hydroxy-2-(*p*-hydroxyphenyl)-benzo(b) thien-3-yl-*p*-(2-(pyrrolidinyl)ethoxy phenyl ketone (LY117018), on endothelial apoptosis and to clarify the mechanisms of action.

* Corresponding author. Department of Geriatric Medicine, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. Tel: +81 3 5800 8830; fax: +81 3 5800 6529.

E-mail address: youchi-tky@umin.ac.jp (Y. Ouchi).

2. Materials and methods

2.1. Chemicals and reagents

The raloxifene analogue LY117018 was provided by Eli-Lilly (Indianapolis, IN, USA). 1,3,5(10)-estratriene-3,17 β -diol (17 β -estradiol), wortmannin and Dulbecco's modified Eagle's medium (DMEM) were purchased from Sigma (St. Louis, MO, USA). Phenol red-free Medium199 (M199) was from Gibco (NY, USA). 7 α ,7 β -9(4,4,5,5,5-Pentafluoropentyl) sulfinylnonyl) estra-1,3,5(10)-triene-3,17-diol (ICI 182,780) was purchased from AstraZeneca (Macclesfield, Cheshire, UK). Hydrogen peroxide (H₂O₂ 30% solution) and actinomycin D were obtained from Wako (Osaka, Japan). The mitogen-activated protein/extracellular signal-regulated protein kinase (MEK)1 inhibitor, 2-[2'-amino-3'-methoxyphenyl]-oxanaphthalen-4-one (PD98059), and antibodies against Akt, phospho-Akt (Ser-473), c-Jun N-terminal kinase (JNK), phospho-JNK (Thr183/Tyr185), extracellular signal-regulated protein kinase1/2 (ERK1/2) and phospho-ERK1/2 (Thr202/Tyr204) were purchased from Cell Signaling (Beverly, MA, USA). Antibodies against p38 (A-12) and phospho-p38 (D-8) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The JNK inhibitor anthrax [1, 9-cd] pyrazol-6(2H)-one (SP600125) and the p-38 inhibitor 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl) 1H-imidazole (SB203580) were from Calbiochem (Darmstadt, Germany). Fetal bovine serum (FBS) was from CCT (Sanko Junyaku Co., Ltd., Tokyo, Japan). Charcoal-stripped fetal bovine serum was from MultiSer (ThermoTrace Ltd., Melbourne, Australia). Nitrocellulose membranes were from Amersham (Buckinghamshire, UK). LumiGLO Reserve Chemiluminescent Substrate Kit was from KPL (Gaithersburg, MD, USA). Cell Death Detection ELISA ^{plus} was purchased from Roche (Mannheim, Germany).

2.2. Cell culture

Bovine carotid endothelial cells (BCEC) were provided by Dr. Sudoh and prepared as described previously (Sudoh et al., 2001; Akishita et al., 1998). Cells were cultured in a 37 °C humidified atmosphere of 95% air/5% CO₂ in DMEM containing 10% FBS and 100 units/ml penicillin/100 μ g/ml streptomycin. For all experiments, BCEC were used at passages 5 to 7, and plated at a concentration of 10⁴ cells/ml. Raloxifene experiments were performed with phenol red-free M199. DMSO was used as a solvent for LY117018, 17 β -estradiol, ICI 182,780 and PD98059. DMSO was present at equal concentrations (0.05%) in all groups, including the vehicle group.

2.3. Apoptosis induction

Apoptosis was induced by addition of hydrogen peroxide (H₂O₂). At 70–80% confluence, cells were washed with phosphate-buffered saline (PBS), and then replenished with phenol red-free M199 without serum, and proliferation was stopped. Cells were exposed to 100 μ M H₂O₂ for 1 h after 6 h starvation, washed twice again with PBS (-), then replenished with phenol red-free M199 containing 5% DCC-FBS. In the same experiments, LY117018 or 17 β -estradiol was added for 30 min before H₂O₂ stimulation in the apoptosis assay. In experiments on inhibitors, the inhibitors were added for 60 min before LY117018 addition. After 24 h of stimulation by H₂O₂, cell apoptosis was evaluated.

2.4. Assay of endothelial cell apoptosis (DNA fragmentation assay)

Cell apoptosis was quantified by means of DNA fragmentation, using a photometric enzyme-linked immunosorbent assay (Cell Death Detection ELISA ^{plus}) kit. Cells with each treatment were lysed in 300 μ l lysis buffer, and a fraction of the supernatant was subjected to reaction for 2 h with the immunocomplex of anti-DNA conjugated with peroxidase, which binds to nucleosomal DNA, and antihistone-biotin, which interacts with streptavidin-coated wells in a microtiter plate. At

the end of the incubation, substrate was added, and development was quantified at 405 nm wavelength.

2.5. Western blot analysis

After treatment with reagents, confluent monolayers of cells were washed two times in ice-cold phosphate-buffered saline and lysed with buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton-X, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM Na₃VO₄, 1 μ g/ml leupeptin, and 1 mM PMSF. For Western blot analysis, total cell lysate was subjected to SDS-polyacrylamide gel electrophoresis (PAGE), and proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. The antibodies used in this study were anti-phospho-ERK1/2 (Thr202/Tyr204), anti-ERK1/2, anti-phospho-Akt (Ser473), anti-Akt, anti-phospho-JNK (Thr183/Tyr185), anti-JNK, anti-phospho-p38, and anti-p38. Antibodies were detected by means of a horseradish peroxidase-linked secondary antibody. Immunoreactive bands were visualized using a LumiGLO Reserve Chemiluminescent Substrate Kit and quantified by densitometry in the linear range of NIH image 1.60.

2.6. Statistics

Values are expressed as means \pm S.E.M. Statistical comparisons were performed by ANOVA followed by Fisher's protected least significance difference (PLSD) test. A probability value <0.05 was considered significant.

3. Results

3.1. Effect of LY117018 on endothelial cell apoptosis

On the basis of concentration- and time-response experiments (data not shown), H₂O₂ (100 μ M) was added to BCEC for 1 h to induce apoptosis. BCEC apoptosis induced by H₂O₂ was significantly attenuated by treatment with LY117018 in a concentration-dependent manner (Fig. 1), while LY117018 per se did not show any effect on apoptosis (data not shown).

3.2. Involvement of MEK/ERK pathway in anti-apoptotic action of LY117018

Phosphorylation levels of p38, JNK, ERK1/2, and Akt were examined because these kinases have been shown to regulate apoptosis (Xia et al.,

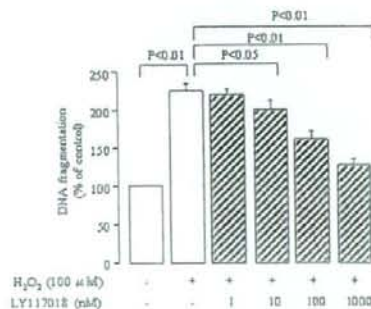


Fig. 1. Effect of LY117018 on H₂O₂-induced endothelial cells apoptosis. At 70–80% confluence, BCEC were starved and exposed to 100 μ M H₂O₂ for 1 h as described in Materials and Methods. Various concentrations of LY117018 (1 nM–1 μ M) were added to the culture medium 30 min before H₂O₂ stimulation in the apoptosis assay. After a 24-h incubation, cell apoptosis was evaluated by means of DNA fragmentation (with a Cell Death Detection ELISA ^{plus} kit) as described in Materials and Methods. Data are expressed as means \pm S.E.M. Differences with a value of $P < 0.05$ were considered statistically significant ($n = 6$).

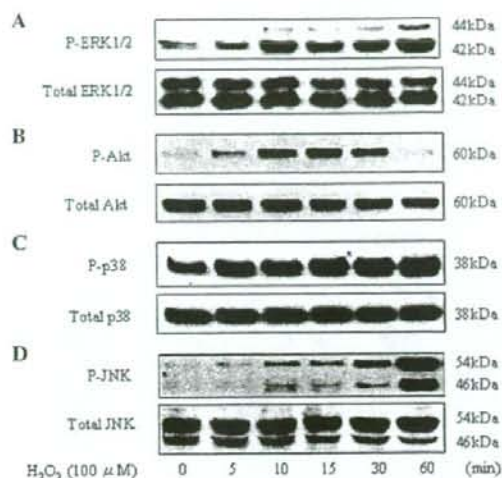


Fig. 2. Phosphorylation of p38, JNK, ERK1/2 and Akt induced by H_2O_2 . Serum-starved cells were stimulated with H_2O_2 (100 μM) and harvested at the times indicated for Western blot analysis. Antibodies against (A) phospho-ERK1/2 (Thr202/Tyr204), ERK1/2, (B) phospho-Akt (Ser473), Akt, (C) phospho-p38, p38, (D) phospho-JNK (Thr183/Tyr185) and JNK were used as described in Materials and methods.

1995; Matsuzaki et al., 1999; Uchiyama et al., 2004). Phosphorylation levels of p38 (D-8), JNK (Thr183/Tyr185), ERK1/2 (Thr202/Tyr204), and Akt (Ser473) were elevated after exposure to H_2O_2 , with no significant

change in the total protein level (Fig. 2A, B, C, D). Maximal phosphorylation was observed at 15 min for Akt (Fig. 2B) and at 60 min for ERK, p38 and JNK (Fig. 2A, C and D).

We examined the effects of a p38 inhibitor, SB203580, and a JNK inhibitor, SP600125, on BCEC apoptosis. BCEC apoptosis was significantly decreased by the inhibitors of p38 and JNK (data not shown). We also examined the effects of a MEK1 (MEK is the immediate upstream regulator of ERK) inhibitor, PD98059, and a phosphatidylinositol-3 OH (PI3) kinase inhibitor, wortmannin, on BCEC apoptosis. PD98059 and wortmannin significantly enhanced H_2O_2 -induced BCEC apoptosis (data not shown). 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. The induction of apoptosis by H_2O_2 may be regulated by the balance between death signaling and survival signaling.

Next, we examined the effects of LY117018 on the phosphorylation levels of p38, JNK, ERK1/2, and Akt. On the basis of time-response experiments (Fig. 2A, B, C, D), cells were stimulated with 100 μM H_2O_2 for 15 min for examination of Akt activity and for 60 min for examination of ERK1/2, p38 and JNK activity. Cells were pretreated with LY117018 for 30 min prior to exposure to H_2O_2 . LY117018 significantly enhanced the phosphorylation level of ERK1/2 (Fig. 3A). However, no change in the phosphorylation of Akt (Fig. 3B), p38 (Fig. 3C) and JNK (Fig. 3D) was induced by LY117018.

We examined the effects of PD98059 on the anti-apoptotic activity of LY117018. The anti-apoptotic effect of LY117018 was prevented by PD98059 (Fig. 3E), while PD98059 alone did not induce BCEC apoptosis. These results suggest that the anti-apoptotic effect of LY117018 was not exerted by inhibition of cell death signals such as p38 or JNK, or by activation of a survival signal, PI3-kinase/Akt, but

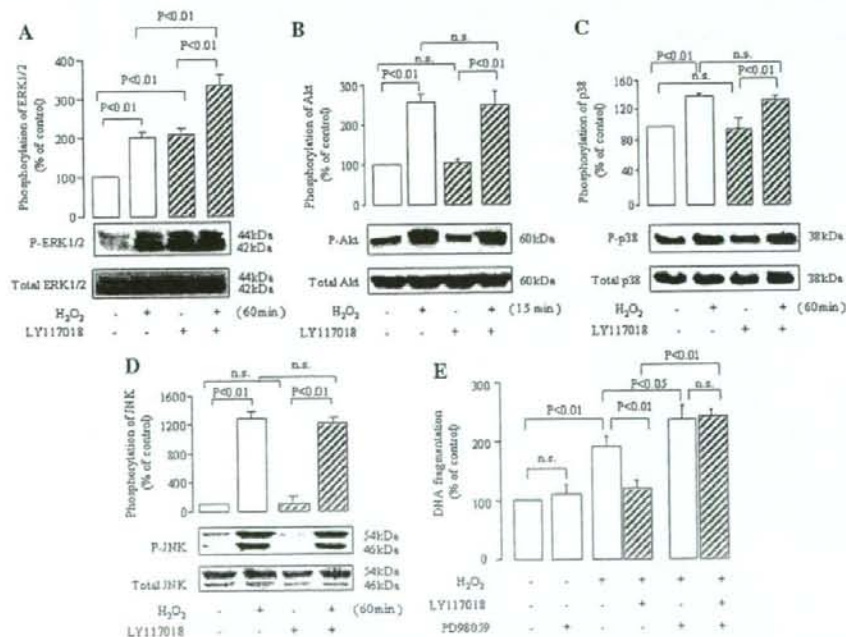


Fig. 3. Effect of LY117018 on H_2O_2 -induced activation of p38, JNK, ERK1/2 and Akt. Serum-starved cells were pretreated with 1 μM LY117018 for 30 min. Then cells were stimulated with H_2O_2 (100 μM) for 15 min for determination of Akt activity (B) and for 60 min for determination of ERK1/2, p38 and JNK (A, C and D) activity. Cells were harvested, lysed and used for Western blot analysis. The activities of ERK1/2 (Thr202/Tyr204), Akt (Ser473), p38 (D-8) and JNK (Thr183/Tyr185) were measured as described in Materials and methods. Representative blots and quantitative data evaluated by densitometry are shown ($n=3$). The data are expressed as means \pm S.E.M. Differences with a value of $P<0.05$ were considered statistically significant. (E) In the PD98059 experiment, cells were pretreated with PD98059 (10 μM) for 1 h before addition of LY117018 (1 μM , 30 min), then stimulated with 100 μM H_2O_2 for 1 h. After a 24-h incubation, cell apoptosis was evaluated by means of DNA fragmentation as described in Materials and methods. Values are expressed as means \pm S.E.M. Differences with a value of $P<0.05$ were considered statistically significant ($n=6$).

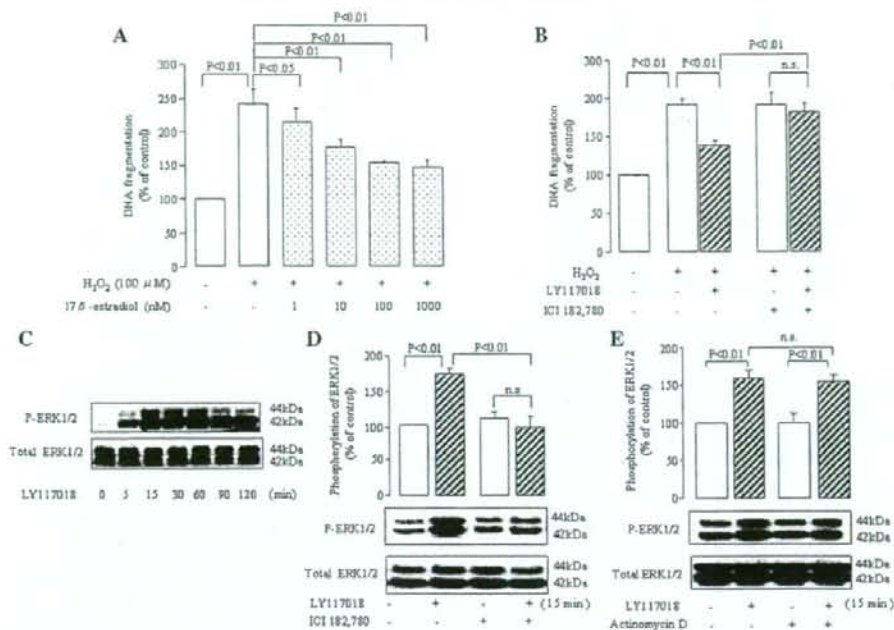


Fig. 4. Involvement of estrogen receptors in anti-apoptotic action of LY117018. At 70–80% confluence, BCEC were starved and exposed to H₂O₂ (100 μM) for 1 h as described in Materials and methods (A and B). (A) Various concentrations of 17β-estradiol (1 nM–1 μM) were added to the culture medium 30 min prior to exposure to H₂O₂ 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 H₂O₂ 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 H₂O₂ (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 H₂O₂. This inhibitory

effect of LY117018 was concentration dependent. LY117018 at 10 nM protected endothelial cells from apoptosis by H₂O₂, 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.

Acknowledgments

This work was supported in part by a Grant-in-Aid (No. 15390-239) for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology. We thank Eli-Lilly (Indianapolis, IN, USA) for providing LY117018.

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., Hozowski, 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, 1045–1055.

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., Mascarelli, 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., Toyama, 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. Neckar 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., Aiko, 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., Engelmann, R.M., Maulik, N., Das, D.K., 2004. Role of Akt signaling in mitochondrial survival pathway triggered by hypoxic preconditioning. *Circulation* 110, 3042–3049.

Wijayarathne, 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 androgens. *Endocrinology* 140, 5828–5840.

Xia, Z., Dickens, M., Ringeaud, J., Davis, R.J., Greenberg, M.E., 1995. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* 270, 1325–1331.

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

Bo-Kyung Son, Masahiro Akishita, Katsuya Iijima, Koichi Kozaki, Koji Maemura, Masato Eto, and Yasuyoshi Ouchi

Department of Geriatric Medicine (B.-K.S., M.A., K.I., M.E., Y.O.), the Department of Cardiovascular Medicine (K.M.), Graduate School of Medicine, The University of Tokyo, Tokyo 113-8655, Japan; and the Department of Geriatric Medicine (K.K.), School of Medicine, The University of Kyorin, Tokyo 181-8611, Japan

Adiponectin exhibits diverse protective effects against atherogenesis and antagonizes many effects of TNF α . Here, we investigated the effect of adiponectin and TNF α on vascular calcification, a critical event in the development and progression of vascular disease. In human aortic smooth muscle cells (HASMC), TNF α augmented inorganic phosphate (Pi)-induced calcification, whereas adiponectin significantly suppressed it and abolished the stimulatory effect of TNF α in a concentration-dependent manner. Similarly, adiponectin ameliorated the accelerating effect of TNF α on Pi-induced apoptosis, the essential process of HASMC calcification. Furthermore, these effects of TNF α and adiponectin were associated with AMP-activated protein kinase (AMPK)-dependent growth arrest-specific gene 6 (Gas6) expression and Akt sig-

naling. 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 TNF α , and the effect of adiponectin was abrogated by compound C. These results demonstrate that adiponectin antagonizes the stimulatory effect of TNF α 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 calcifica-

tion in type 1 diabetic and nondiabetic subjects, independent of other cardiovascular risk factors (13). Experimental studies have shown that adiponectin reduces TNF α production in response to various stresses, whereas TNF α 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 TNF α 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.

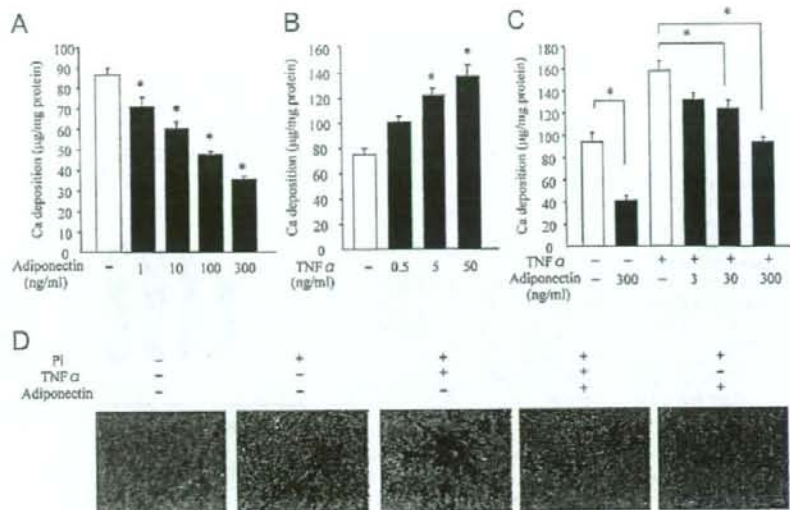
In the present study, we investigated whether adiponectin and TNF α modulate Pi-induced VSMC calcification by regulating apoptosis. We found that TNF α had a stimulatory effect, whereas adiponectin had an inhibitory effect on Pi-induced apoptosis and calcification in human aortic smooth muscle cells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

First Published Online January 3, 2008

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.

Endocrinology is published monthly by The Endocrine Society (<http://www.endo-society.org>), the foremost professional society serving the endocrine community.

FIG. 1. Effect of adiponectin and $TNF\alpha$ on Pi-induced calcification. A and B, HASMC were cultured with the indicated concentrations of adiponectin (A) or $TNF\alpha$ (B) in calcification medium. They were added simultaneously when the medium was changed every 2 d. C, The effect of $TNF\alpha$ (20 ng/ml) and adiponectin with the indicated concentrations on Ca deposition was determined at 6 d. D, The effect of $TNF\alpha$ (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 \pm 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% CO₂. HASMC were used up to passage 8 for the experiments.

Induction and quantification of calcification

For Pi-induced calcification, Pi (a mixed solution of Na₂HPO₄ and NaH₂PO₄ whose pH was adjusted to 7.4) was added to serum-supple-

mented 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 $TNF\alpha$ (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, Mannheim, Germany) and ter-

FIG. 2. Effect of adiponectin and $TNF\alpha$ 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 $TNF\alpha$ (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 $TNF\alpha$ (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 \pm SE (n = 3). *, $P < 0.05$ by Bonferroni test. Each experiment was performed in triplicate for each condition.

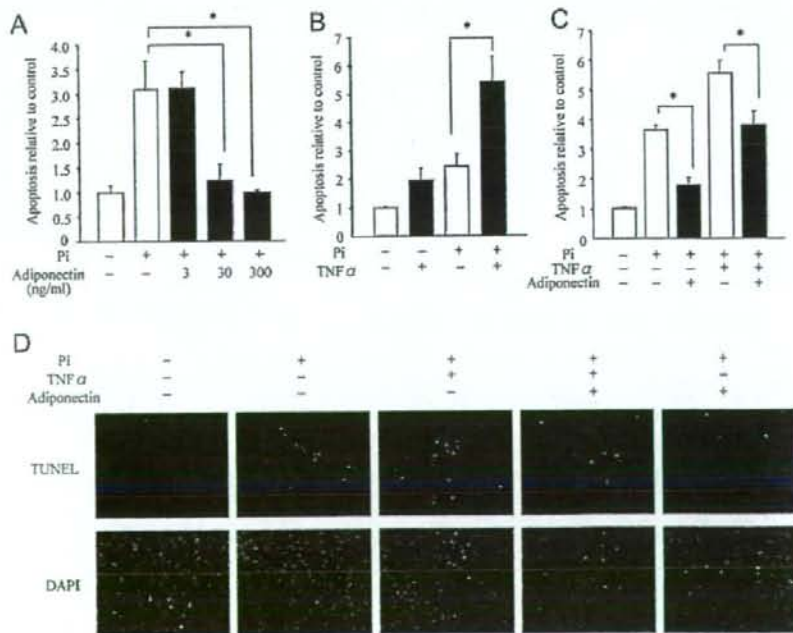
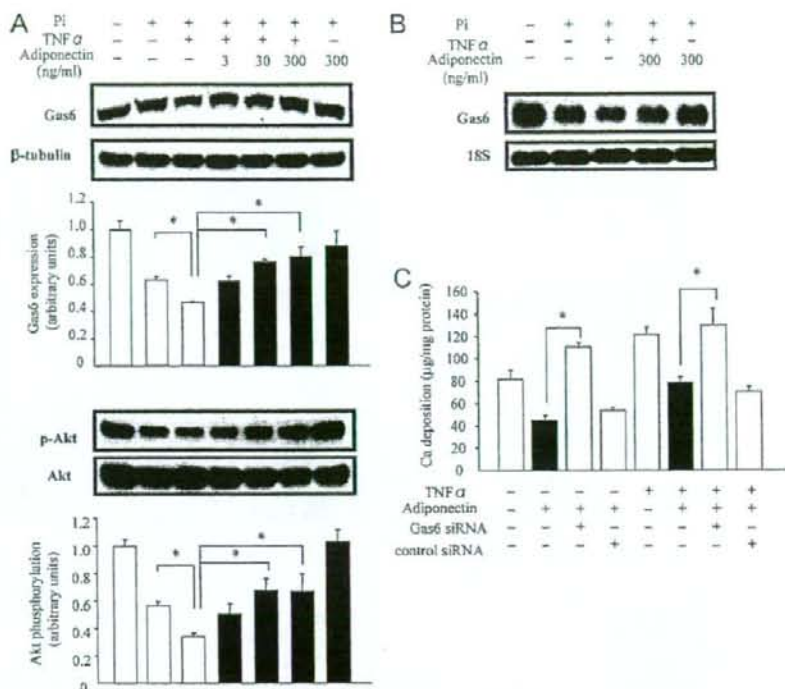


FIG. 3. Gas6 is the target of the effect of adiponectin and TNF α on Pi-induced calcification. HASMC were cultured with the indicated concentrations of adiponectin and TNF α (20 ng/ml). On d 6, cell lysates were collected and immunoblotted with antibodies that recognize Gas6, phospho-Akt (p-Akt), Akt, or β -tubulin. A, The untreated condition is the serum-supplemented status without Pi. B, Total RNA (5 μ g) was harvested for Northern blot analysis after HASMC were incubated with adiponectin (300 ng/ml) and TNF α (20 ng/ml) for 6 d. When HASMC had reached 80–90% confluence, siRNA (100 nM) was transfected and then was transfected every 2 d with adiponectin (300 ng/ml) and TNF α (20 ng/ml) up to 6 d. C, Ca deposition was measured and normalized by cell protein content. All values are presented as mean \pm SE (n = 3). *, P < 0.05 by Bonferroni test. Each experiment was performed in triplicate for each condition.



minimal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay with ApopTag Plus obtained from Chemicon International, Ltd. (Hampshire, UK), according to the manufacturer's instructions.

Generation of promoter reporter construct and luciferase activity assay

The 1925-bp Gas6 promoter (-1827/+99) corresponding to the Gas6 promoter sequences was generated by PCR from human genomic DNA with the appropriate sets of primers (6). These inserts were cloned into a pGL3 basic vector (Promega, Charbonnières, France) by standard molecular biological techniques. The construct was verified by sequencing. HASMC were transiently transfected in 12-well plates with 0.8 μ g plasmid DNA and lipofectamine 2000 (Invitrogen Corp., Paisley, UK) according to the procedure recommended by the manufacturer. Cells were treated with TNF α , adiponectin, and compound C at 24 h after transfection, followed by incubation for an additional 44 h. Firefly luciferase activity was determined using a luciferase assay system (Promega) and normalized by total cell protein.

Preparation of small interfering RNA (siRNA) targeting Gas6 and transfection

To evaluate the role of Gas6 in the inhibitory effect of adiponectin on calcification, we knocked down Gas6 using siRNA. Two kinds of siRNA were designed to target human Gas6 and nonspecific control siRNA was synthesized using standard templates (6). siRNA (100 nM) was transfected using transfection reagent (Upstate, Charlottesville, VA) when HASMC had reached 80–90% confluence and then was transfected every 2 d with TNF α and adiponectin up to 6 d. The efficiency of Gas6 siRNA was confirmed with immunoblotting (6).

RNA extraction and Northern blot analysis

Total RNA was extracted from HASMC using an RNeasy minikit (QIAGEN, Courtaboeuf, France). For Northern blot analysis, harvested RNA (5 μ g) was fractionated on 1.4% formaldehyde-agarose gel and

transferred to a nylon filter. The filter was hybridized at 68 C for 2 h with 32 P-labeled Gas6 cDNA (6) and an 18S probe in QuickHyb solution (Stratagene, La Jolla, CA) and autoradiographed.

Immunoblotting

The effect of TNF α and adiponectin on the expression of Gas6, phospho-Akt, and Akt was examined, as described previously (24). Analysis of AMPK activation was performed using an antibody specific for the phosphorylated Thr172 of AMPK (Cell Signaling Technology Inc., Beverly, MA).

Statistical analysis

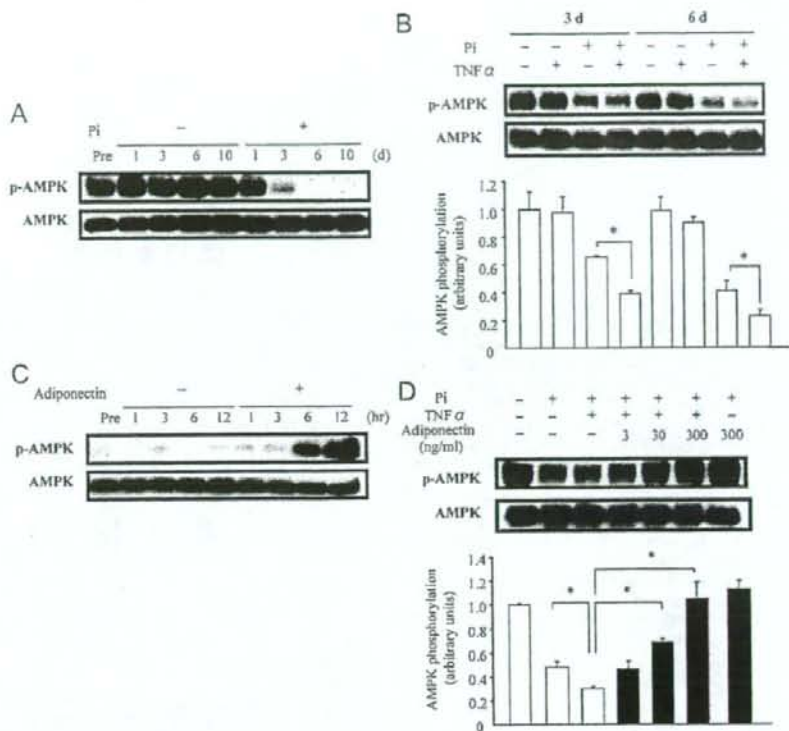
All results are presented as mean \pm SE. Statistical comparisons were made by ANOVA, followed by Bonferroni test. A value of P < 0.05 was considered statistically significant.

Results

Adiponectin and TNF α regulate Pi-induced calcification in HASMC

To investigate the effect of adiponectin and TNF α on Pi-induced calcification, HASMC were incubated with adiponectin and TNF α in the presence of 2.6 mM Pi. On d 6, Ca deposition was suppressed by adiponectin in a concentration-dependent manner (40 \pm 2% of control at 300 ng/ml, Fig. 1A), whereas TNF α significantly augmented Ca deposition (182 \pm 13% of control at 50 ng/ml; Fig. 1B). Furthermore, adiponectin clearly inhibited Ca deposition stimulated by TNF α in a concentration-dependent manner (Fig. 1C). This was also found by von Kossa's staining (Fig. 1D). These results suggest that adiponectin has an inhibitory effect on both Pi-induced and TNF α -stimulated calcification in HASMC.

FIG. 4. Effect of adiponectin and TNF α on AMPK activity during Pi-induced calcification. HASMC were cultured in the absence or presence of Pi (2.6 mM) for up to 10 d. After the indicated incubation period, cell lysates were harvested and immunoblotted with antibodies to phospho-AMPK (p-AMPK) and AMPK. **A**, The untreated condition is the serum-supplemented status without Pi. **B**, Immunoblotting analysis showing the effect of TNF α (20 ng/ml) on p-AMPK and AMPK expression in the absence or presence of serum containing Pi (2.6 mM). **C**, Serum-starved HASMC were incubated with or without adiponectin (300 ng/ml) for 12 h. HASMC were cultured with the indicated concentrations of adiponectin and TNF α (20 ng/ml). **D**, On d 6, cell lysates were harvested and immunoblotted with antibodies to p-AMPK and AMPK. All values are presented as mean \pm SE (n = 3). **P* < 0.05 by Bonferroni test. Each experiment was performed in triplicate for each condition.



Adiponectin antagonizes stimulatory effect of TNF α on Pi-induced apoptosis by restoration of Gas6-mediated survival pathway

Because apoptosis has been shown to be an important pathway regulating Pi-induced calcification (6, 24), we examined the effect of adiponectin and TNF α on apoptosis in HASMC. Adiponectin, at concentrations exerting inhibitory effects on calcification, significantly reduced apoptosis, as quantified by cytoplasmic histone-associated DNA fragments (Fig. 2A). On the other hand, apoptosis was enhanced by TNF α in the presence of Pi (Fig. 2B). As shown in Ca deposition, adiponectin antagonized the stimulatory effect of TNF α on apoptosis. This inhibition was also observed by TUNEL assay (Fig. 2, C and D).

We previously demonstrated that Pi-induced apoptosis was mediated by down-regulation of the Gas6-mediated survival pathway (6, 24). Therefore, we examined the effects of adiponectin and TNF α on this pathway. Both Gas6 mRNA and protein expression were down-regulated by TNF α in the presence of Pi, whereas adiponectin clearly restored their expression (Fig. 3, A and B). Next, because the Gas6-mediated survival pathway is Akt-dependent, the effect of adiponectin and TNF α on Akt phosphorylation was examined. As shown in the Gas6 expression, the similar effect of adiponectin and TNF α was observed in Akt phosphorylation that is high at basal level in the untreated condition containing serum (Fig. 3A). We confirmed that total Akt was not changed by adiponectin and

TNF α treatment (Fig. 3A). On the other hand, adiponectin and TNF α did not affect Gas6 expression and Akt phosphorylation in the condition without Pi treatment (data not shown).

Furthermore, to evaluate the role of Gas6 in the inhibitory effect of adiponectin on calcification, we examined whether the knockdown of Gas6 abrogated the effects of adiponectin using siRNA. On d 6, transfection of Gas6 siRNA markedly decreased its expression (data not shown), as reported previously (6). The inhibitory effect of adiponectin on Pi- and TNF α -induced calcification was reversed by Gas6 siRNA, supporting the critical role of Gas6 in the effect of adiponectin on calcification (Fig. 3C).

AMPK plays a critical role in VSMC calcification and is regulated by adiponectin and TNF α

It has been reported that AMPK is a central signaling molecule in adiponectin's action (19, 20). We investigated whether AMPK is involved in the effect of adiponectin on Pi-induced calcification. First, we examined the activity of AMPK during calcification. Immunoblot analysis showed that phosphorylated AMPK was markedly down-regulated in the presence of Pi for 10 d, whereas the expression of total AMPK was not changed (Fig. 4A). TNF α further inhibited its phosphorylation in the presence of Pi, without changing total AMPK (Fig. 4B). In the case of adiponectin, AMPK phosphorylation was remarkably stimulated in a time-dependent manner (Fig. 4C). As shown in Fig. 4D, adiponectin further restored AMPK phos-