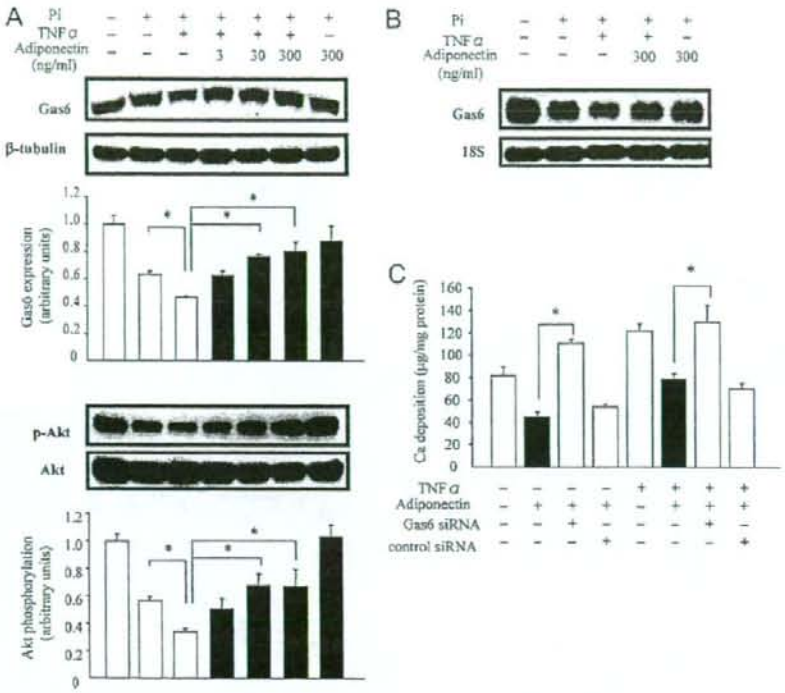

FIG. 3. Gas6 is the target of the effect of adiponectin and TNFα on Pi-induced calcification. HASMC were cultured with the indicated concentrations of adiponectin and TNFα (20 ng/ml). On d 6, cell lysates were collected and immunoblotted with antibodies that recognize Gas6, phospho-Akt (p-Akt), Akt, or β-tubulin. A, The untreated condition is the serum-supplemented status without Pi. B, Total RNA (5 μg) was harvested for Northern blot analysis after HASMC were incubated with adiponectin (300 ng/ml) and TNFα (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 TNFα (20 ng/ml) up to 6 d. C, Ca deposition was measured and normalized by cell protein content. All values are presented as mean ± SE (n = 3). *, $P < 0.05$ by Bonferroni test. Each experiment was performed in triplicate for each condition.

minal 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 μg plasmid DNA and lipofectamine 2000 (Invitrogen Corp., Paisley, UK) according to the procedure recommended by the manufacturer. Cells were treated with TNFα, 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 TNFα 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 μg) 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 TNFα 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 ± SE. Statistical comparisons were made by ANOVA, followed by Bonferroni test. A value of $P < 0.05$ was considered statistically significant.

Results

Adiponectin and TNFα regulate Pi-induced calcification in HASMC

To investigate the effect of adiponectin and TNFα on Pi-induced calcification, HASMC were incubated with adiponectin and TNFα in the presence of 2.6 mM Pi. On d 6, Ca deposition was suppressed by adiponectin in a concentration-dependent manner (40 ± 2% of control at 300 ng/ml, Fig. 1A), whereas TNFα significantly augmented Ca deposition (182 ± 13% of control at 50 ng/ml; Fig. 1B). Furthermore, adiponectin clearly inhibited Ca deposition stimulated by TNFα 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 TNFα-stimulated calcification in HASMC.

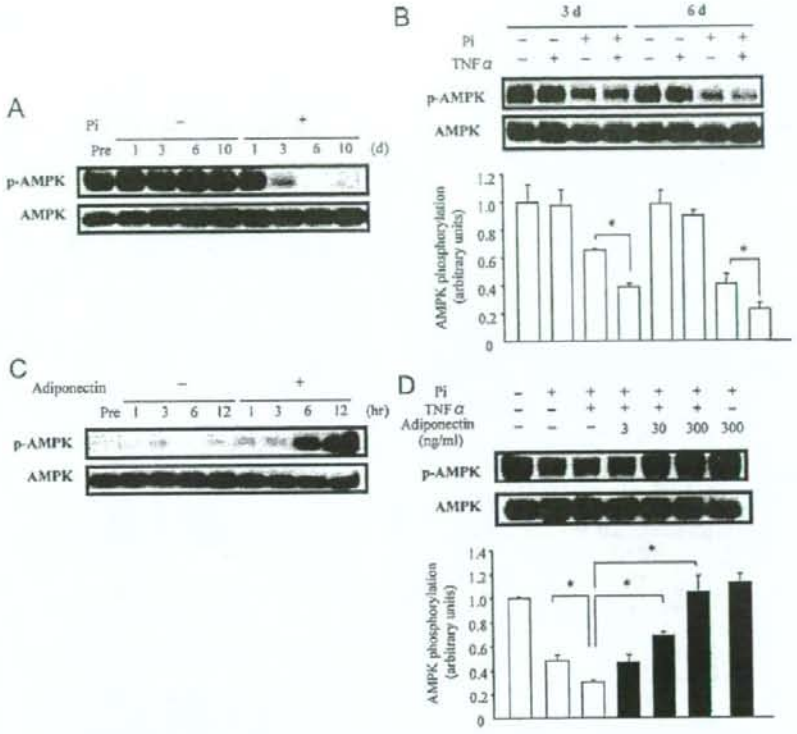

FIG. 4. Effect of adiponectin and TNFα on AMPK activity during Pi-induced calcification. HASMC were cultured in the absence or presence of Pi (2.6 mM) for up to 10 d. After the indicated incubation period, cell lysates were harvested and immunoblotted with antibodies to phospho-AMPK (p-AMPK) and AMPK. A, The untreated condition is the serum-supplemented status without Pi. B, Immunoblotting analysis showing the effect of TNFα (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 TNFα (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 ± SE (n = 3). *, $P < 0.05$ by Bonferroni test. Each experiment was performed in triplicate for each condition.

Adiponectin antagonizes stimulatory effect of TNFα 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 TNFα 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 TNFα in the presence of Pi (Fig. 2B). As shown in Ca deposition, adiponectin antagonized the stimulatory effect of TNFα 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 TNFα on this pathway. Both Gas6 mRNA and protein expression were down-regulated by TNFα 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 TNFα on Akt phosphorylation was examined. As shown in the Gas6 expression, the similar effect of adiponectin and TNFα 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 TNFα treatment (Fig. 3A). On the other hand, adiponectin and TNFα 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 TNFα-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 TNFα

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). TNFα 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-

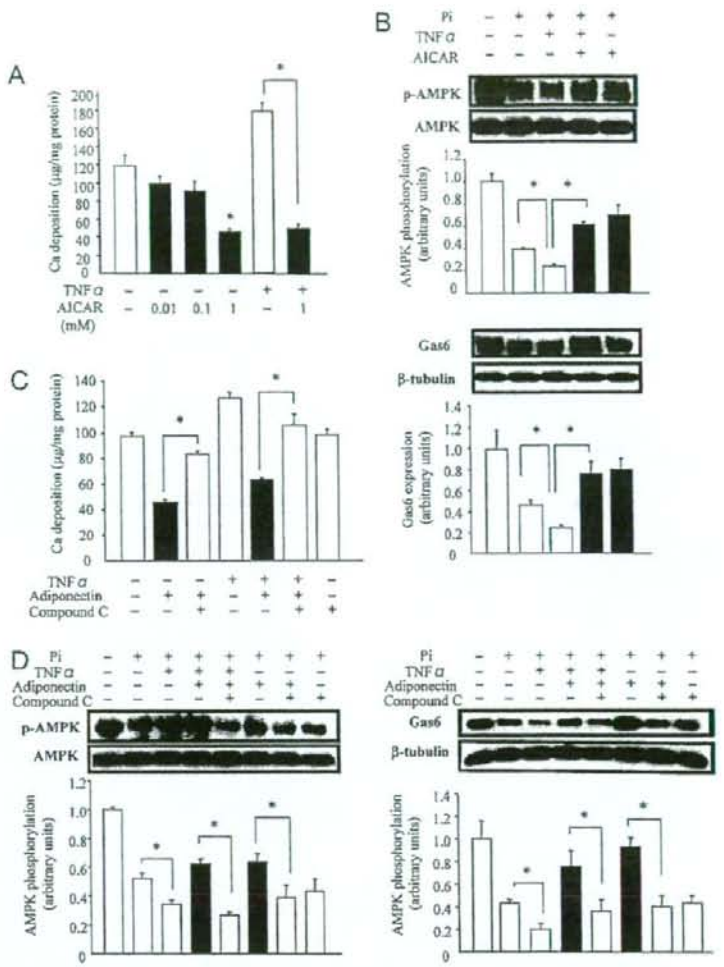

FIG. 5. AMPK plays an important role in Pi-induced calcification. HASMC were treated with or without AICAR (1 mM), a pharmacological activator of AMPK and TNFα (20 ng/ml) in calcification medium for 6 d. A and B, Ca deposition (n = 6) (A) was measured, and immunoblotting with antibodies to p-AMPK, AMPK, Gas6, and β-tubulin (B) was performed (n = 3). HASMC were cultured with or without compound C (1 μM), a chemical inhibitor of AMPK, adiponectin (300 ng/ml), and TNFα (20 ng/ml) in calcification medium for 6 d. C and D, Ca deposition (C) was evaluated (n = 6), and immunoblotting with antibodies to p-AMPK, AMPK, Gas6, and β-tubulin (D) was performed (n = 3). All values are presented as mean ± SE. *, $P < 0.05$ by Bonferroni test. Each experiment was performed in triplicate for each condition.

phorylation that was inhibited by Pi and TNFα in a calcification-promoting condition.

To clarify the causal relationship between AMPK and calcification, we tried to activate AMPK by treatment with 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) (25). In HASMC, AICAR significantly inhibited Ca deposition in a concentration-dependent manner (Fig. 5A). In addition, TNFα-stimulated Ca deposition was also blunted by AICAR. Interestingly, AICAR restored Gas6 expression down-regulated by Pi and TNFα (Fig. 5B). Next, to investigate whether the effect of adiponectin is dependent on AMPK, we tried to block AMPK using compound C, a chemical inhibitor of AMPK. As shown in Fig. 5C, compound C clearly abrogated the inhibitory effect of adiponectin both on Pi- and TNFα-induced calcification. The increase in Gas6 expression as well as AMPK phosphorylation by adiponectin was also abolished by compound C (Fig. 5D). These results suggest that AMPK regulates Gas6 expression, followed by regulation of Ca deposition in HASMC.

Transcription activity of Gas6 is regulated by adiponectin and TNFα via AMPK

To investigate whether Gas6 expression is transcriptionally regulated by adiponectin, TNFα, and AMPK, a promoter study was undertaken. Reporter assay using the −1.9-kb Gas6-luciferase DNA construct revealed that adiponectin completely reversed the down-regulation of Gas6 transcription activity by TNFα. Furthermore, compound C abrogated the effect of adiponectin on Gas6 transcription activity, indicating that adiponectin and TNFα regulate Gas6 expression at the transcription level via AMPK activity (Fig. 6).

Discussion

The present study showed that adiponectin has a protective effect against Pi-induced calcification and, furthermore,

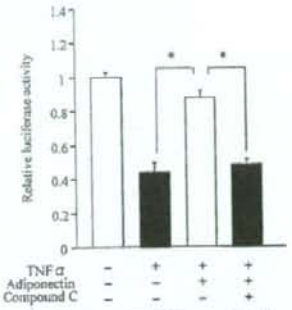

FIG. 6. Effect of adiponectin and TNFα on Gas6 promoter activity. HASMC were transfected with the Gas6 promoter-luciferase construct using lipofectamine 2000. Twenty-four hours after transfection, adiponectin (300 ng/ml), compound C (1 μM), and TNFα (20 ng/ml) were added. Cells were incubated for an additional 44 h. Luciferase activity was normalized to that of vehicle-treated cells. All values are presented as mean ± SE (n = 4). *, $P < 0.05$ by Bonferroni test. Each experiment was performed in triplicate for each condition.

has an antagonistic effect on TNFα-augmented calcification. Based on our previous finding that Pi-induced calcification is dependent on apoptotic cell death in HASMC, we examined the role of adiponectin and TNFα in Pi-induced apoptosis. As expected, we found that adiponectin had an inhibitory effect and TNFα had a stimulatory effect on Pi-induced apoptosis. This study also demonstrated the

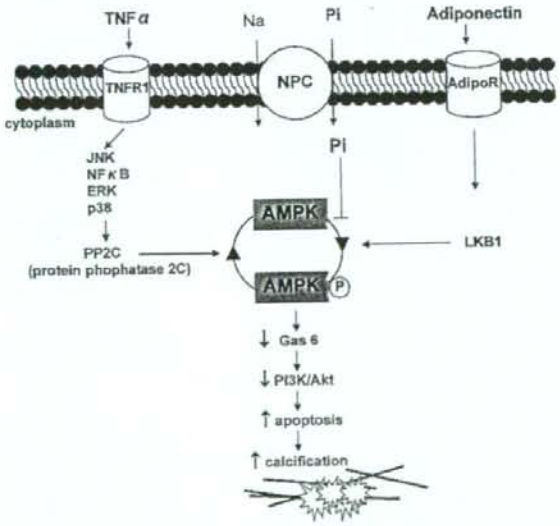

FIG. 7. Scheme of the effect of TNFα and adiponectin on Pi-induced calcification. In HASMC, exogenous Pi is internalized by sodium-dependent phosphate cotransporter (NPC, such as Pit-1) and inhibits AMPK phosphorylation, followed by down-regulation of the Gas6-mediated survival pathway. This pathway stimulates apoptosis, leading to subsequent development of calcification. TNFα directly suppresses AMPK activation by promoting PP2C activation via TNF receptor-1 (TNFR1). On the other hand, adiponectin activates LKB1-AMPK pathway via adiponectin receptors (AdipoR). AMPK activation modulated by TNFα and adiponectin contributes to the regulation of Pi-induced calcification.

regulation of Gas6 expression by TNFα and adiponectin, a suppressive effect and a promoting effect, respectively, at the transcriptional level. Akt, a critical downstream effecter of Gas6, was activated by adiponectin, whereas TNFα had an opposite action on its phosphorylation. Given that adiponectin and TNFα did not affect Gas6 expression and Akt phosphorylation in the absence of Pi (data not shown), the effects of adiponectin and TNFα on these molecules may dependent on Pi-induced responses. These results suggest that Gas6 is the target of adiponectin and TNFα in regulating Pi-induced apoptosis, accompanied by modulation of the Akt-dependent survival pathway.

As reported previously (6), Pi-induced VSMC calcification is associated with both phenotypic transition to osteoblastic cells via sodium-dependent phosphate cotransporter and apoptotic cell death. In our preliminary experiments, the expression of osteopontin, an osteoblastic marker, was not affected by TNFα and adiponectin (data not shown). Although this result suggests little influence of TNFα and adiponectin on osteoblastic differentiation of VSMC, extensive and systematic investigation including other markers of osteoblastic differentiation is needed to conclude this issue.

Multiple lines of clinical evidence show that adiponectin has protective actions on the cardiovascular system (26, 27). Circulating levels of adiponectin in humans are as high as 500–30,000 μg/ml (28). Therefore, the concentration of adiponectin (300 ng/ml) used in this study are within physiological levels. Especially, consistent with our findings, adiponectin has been implicated in apoptosis of cardiovascular cells (19, 23, 29). Adiponectin inhibits apoptosis in cardiac myocytes and fibroblasts that are exposed to hypoxia-reoxygenation stress (19). In endothelial cells, adiponectin has been reported to inhibit serum starvation-induced apoptosis (23). *In vivo* experiments have also shown that adiponectin-deficient mice develop larger myocardial infarcts due to increased myocardial cell apoptosis and TNFα expression (17). Taking these observations together with our results, the antiapoptotic actions of adiponectin contribute to the inhibition of VSMC calcification.

Most effects of adiponectin have been attributed to the activation of AMPK, which affects many aspects of cellular metabolism including glucose uptake (30, 31), glucose utilization (32), and fatty acid oxidation (33, 34). Recently, AMPK activation in VSMC has been suggested as a target to prevent or treat vascular disease (35, 36). AICAR-induced AMPK activation inhibited angiotensin II-stimulated VSMC proliferation, and administration of AICAR prevented neointimal formation in a rat balloon injury model (35). AMPK activation in VSMC elicited cell cycle arrest at the G1 phase and inhibited cell proliferation via p53 up-regulation (36). Furthermore, in the heart, the inhibitory effects of adiponectin on ischemic injury-induced apoptosis have been shown to be dependent on AMPK activation (19). The results of *in vitro* studies also revealed that AMPK signaling is essential for the antiapoptotic activities of adiponectin on endothelial cells (23). These observations are consistent with the finding of the present study that AMPK activated by adiponectin stimulated Gas6 expression to restore the survival pathway, leading to the suppression of calcification.

In the present study, we further demonstrated that adiponectin significantly augmented the transcriptional activity of Gas6

that was decreased by TNFα. 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 TNFα. 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-κB-regulated transcription, which is activated by TNFα (39).

Consistent with our findings, it has been recently reported that TNFα 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 TNFα 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 TNFα 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 TNFα. 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 TNFα in vascular calcification might be a key to the management of cardiovascular disease.

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Original Article

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

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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±SD: 57±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±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 (β=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 (β=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-

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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 cardiovascular 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. †$p<0.001$, *$p<0.05$.

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. †$p<0.01$.

for estradiol ($n=34$), 0976 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 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 (β=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 (β=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-

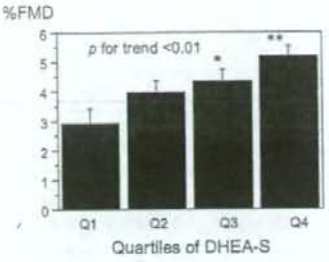

Fig. 1. *Percent flow-mediated dilation of the brachial artery (%FMD) according to quartiles of serum dehydroepiandrosterone-sulfate (DHEA-S). Values are expressed as the means±SEM. *$p<0.05$, **$p<0.01$ vs. Q1.*

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 ± 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.

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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	139.1 ± 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.

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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.

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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.

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

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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, 7α,7β-(9[(4,4,5,5,5-Pentafluoropentyl)sulfinyl]nonyl) estra-1,3,5(10)-triene-3,17-diol (ICI 182,780). 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 182,780. The activation of ERK/1/2 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.

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 (*M*ultiple *O*utcomes of *R*aloxifene *E*valuation) 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.

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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)-estariene-3,17β-diol (17β-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). 7α,7β-(9[(4,4,5,5,5-Pentafluoropentyl) sulfinyl]nonyl) estra-1,3,5(10)-triene-3,17-diol (ICI 182,780) was purchased from AstraZeneca (Macclesfield, Cheshire, UK). Hydrogen peroxide (H_2O_2 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, Gemany). 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% CO_2 in DMEM containing 10% FBS and 100 units/ml penicillin/100 μg/ml streptomycin. For all experiments, BCEC were used at passages 5 to 7, and plated at a concentration of 10^4 cells/ml. Raloxifene experiments were performed with phenol red-free M199. DMSO was used as a solvent for LY117018, 17β-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 (H_2O_2). 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 μM H_2O_2 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 17β-estradiol was added for 30 min before H_2O_2 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 H_2O_2, 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 μl 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 Na_3VO_4, 1 μg/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 polyvinilidene 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 ± 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), H_2O_2 (100 μM) was added to BCEC for 1 h to induce apoptosis. BCEC apoptosis induced by H_2O_2 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 LY 110718

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

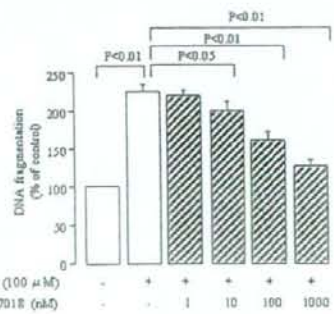

Fig. 1. Effect of LY117018 on H_2O_2-induced endothelial cells apoptosis. At 70–80% confluence, BCEC were starved and exposed to 100 μM H_2O_2 for 1 h as described in Materials and methods. Various concentrations of LY117018 (1 nM–1 μM) were added to the culture medium 30 min before H_2O_2 stimulation 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 ± S.E.M. Differences with a value of $P<0.05$ were considered statistically significant ($n=6$).

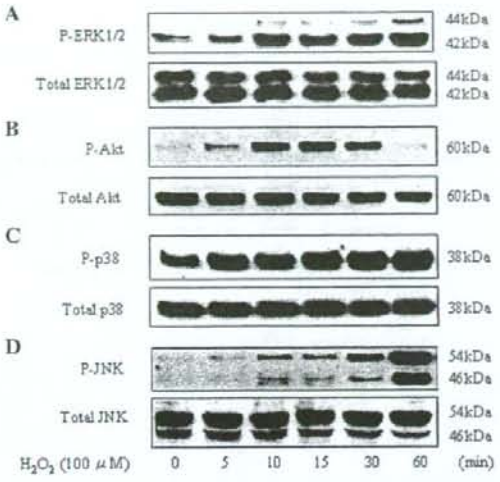

Fig. 2. Phosphorylation of p38, JNK, ERK1/2 and Akt induced by H_2O_2. Serum-starved cells were stimulated with H_2O_2 (100 μM) and harvested at the times indicated for Western blot analysis. Antibodies against (A) phospho-ERK1/2 (Thr202/Tyr204), ERK1/2, (B) phospho-Akt (Ser473), Akt, (C) phopho-p38, 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-kianse/Akt, but

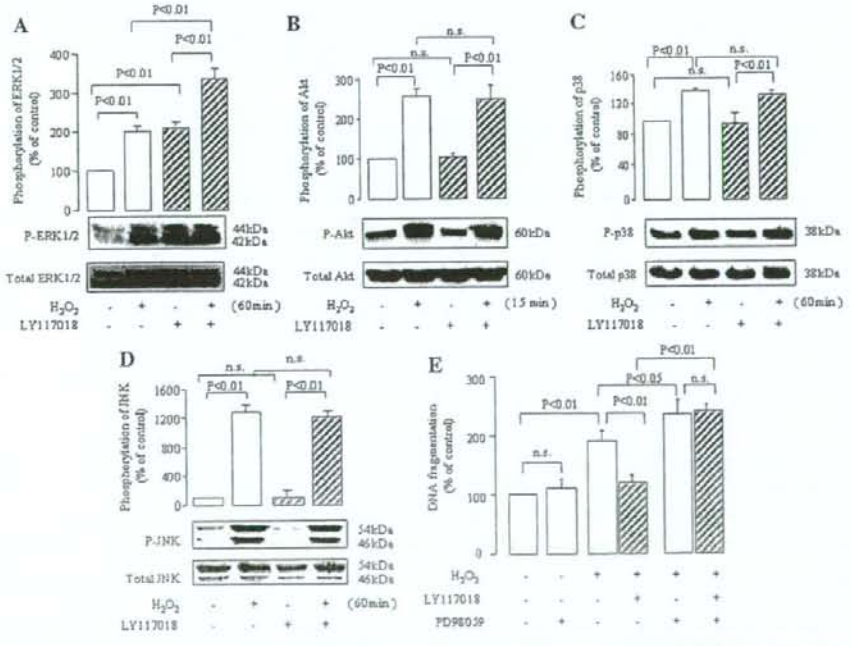

Fig. 3. Effect of LY117018 on H_2O_2-induced activation of p38, JNK, ERK1/2 and Akt. Serum-starved cells were pretreated with 1 μM LY117018 for 30 min. Then cells were stimulated with H_2O_2 (100 μM) for 15 min for determination of Akt activity (B) and for 60 min for determination of ERK1/2, p38 and JNK (A, C and D) activity. Cells were harvested, lysed and used for Western blot analysis. The activities of ERK1/2 (Thr202/Thr204), 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 ± 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 ± S.E.M. Differences with a value of $P<0.05$ were considered statistically significant ($n=6$).

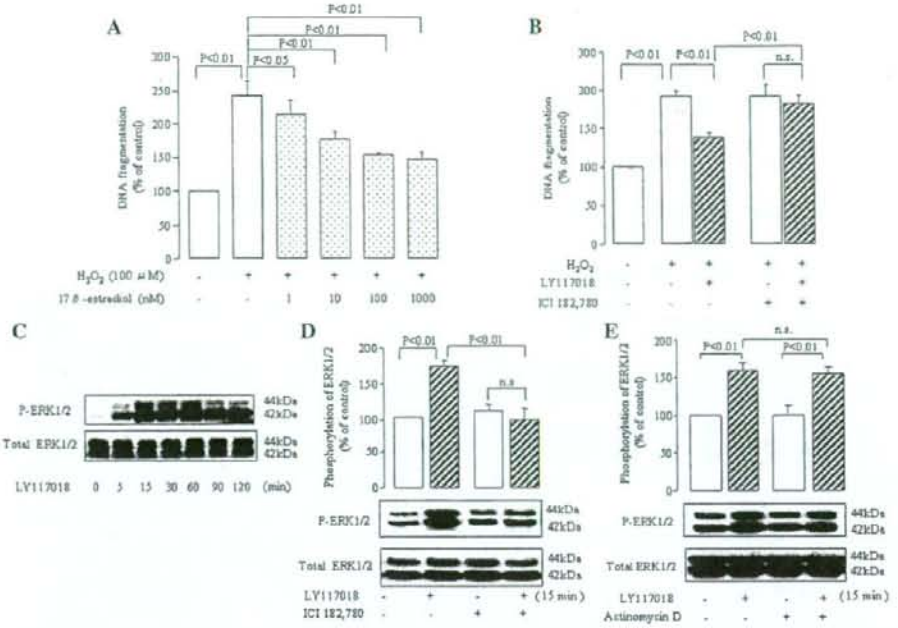

Fig. 4. Involvement of estrogen receptors in anti-apoptotic action of LY117018. At 70–80% confluence, BCEC were starved and exposed to H_2O_2 (100 μM) for 1 h as described in Materials and methods (A and B). (A) Various concentrations of 17β-estradiol (1 nM–1 μM) were added to the culture medium 30 min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

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

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

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

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

4. Discussion

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

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

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

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

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

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Appendix A. Supplementary data

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

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