られた。さらに，表2の性腺機能の欄に示したように，性腺機能低下と効果の有無の間には特に関連はみいだされなかった。

おわりに

以上，アンドロゲン補充療法が脳機能に及ぼす影響について，認知機能とうつ病・うつ状態への影響に絞って説明してきた。どちらに関しても，おおむねポジティブな結果がでており，特に性腺機能低下が認められる高齢男性や難治性のうつ病患者では，もっと積極的に適応が考えられてもよさそうである。またジェルのテストステロン製剤で十分に効果が認められていることから，我が国でも使用できるようになることが望まれる。

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Original Article

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

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

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

Introduction

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

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

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Table 1. Baseline Clinical Characteristics

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

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

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

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

Methods

Patients

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

Twenty-Four-Hour ABPM

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

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

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

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

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

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

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

Follow-Up

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

Data Analysis

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

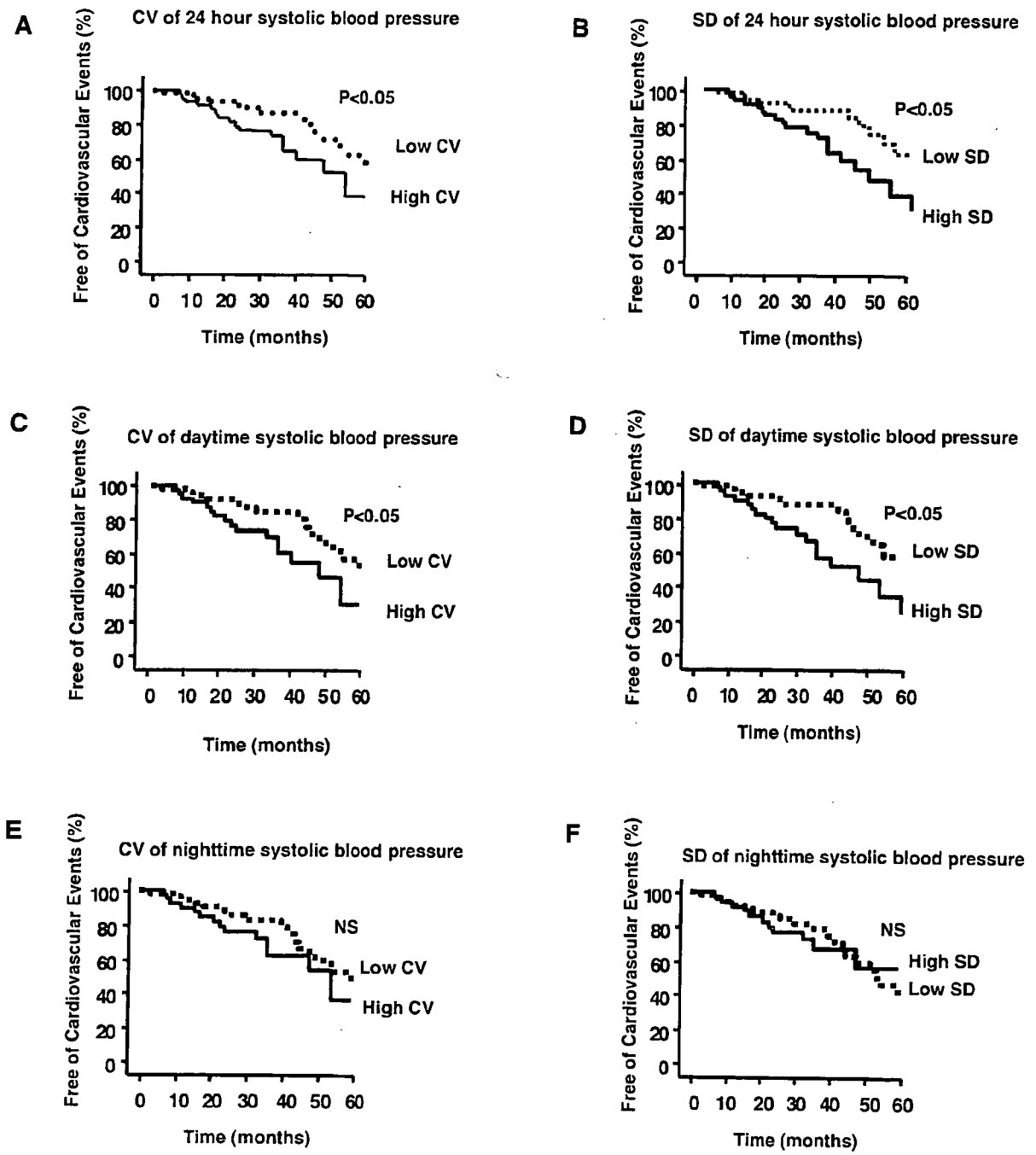

Fig. 1. *Cumulative event-free rates of cardiovascular events. Patients were divided into two groups according to the mean values of the CV of 24-h blood pressure (A), daytime blood pressure (C) or nighttime blood pressure (E), or those of the SD of 24-h blood pressure (B), daytime blood pressure (D)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

Results

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

Table 3. Relative Risk of Cardiovascuiar Events

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

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

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

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

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

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

Discussion

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

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

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

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

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

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

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

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

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

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

Stress-induced injury of vascular endothelial cells (ECs) is considered to be an initial event in the development of atherosclerosis.[1] In particular, oxidative stress has been implicated in endothelial injury caused by oxidized LDL and smoking, as well as hypertension, diabetes, and ischemia reperfusion.[1–3] This notion is supported by the findings that the production of reactive oxygen species is upregulated in vascular lesions[4,5] and that lesion formation such as endothelial dysfunction is accelerated by superoxide anion[6] and, in contrast, is attenuated by free radical scavengers, including vitamin E[7] and superoxide dismutase.[8]

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

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

Methods

H_2O_2 Treatment of Carotid Artery

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

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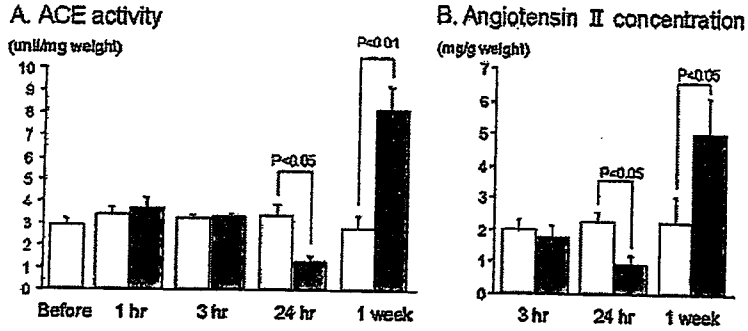

Figure 1. ACE activity and Ang II concentration in rat carotid artery after H_2O_2 treatment. Treated (closed bars) and contralateral (open bar) carotid arteries were harvested at the indicated time points after H_2O_2 treatment. ACE activity and Ang II concentration in tissue homogenates were measured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

Animal Groups and Blood Pressure Measurement

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

Measurement of ACE Activity and Ang II Concentration

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

Evaluation of EC Apoptosis and Neointimal Formation in Carotid Artery

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

Induction of EC Apoptosis in Culture

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

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

Measurement of EC Apoptosis and Oxidative Stress Markers in Culture

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

Formation of 8-isoprostane (8-*iso* prostaglandin $F_{2\alpha}$) was measured using a commercially available EIA kit (Cayman Chemical). Culture supernatants were diluted with EIA buffer when necessary and were applied to EIA according to manufacturer instructions. Intracellular oxidative stress levels were measured using 2',7'-dichlorofluorescein (DCF) as described previously,[26] and the intensity values were calculated using the Metamorph software.

Real-Time Polymerase Chain Reaction

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

Data Analysis

The values are expressed as mean±SEM in the text and figure data were analyzed using 1-factor ANOVA. If a statistically significant effect was found, Newman–Keuls test was performed to isolate the difference between the groups. Differences with a value of $P<0.05$ were considered statistically significant.

Results

ACE Activity in Carotid Artery After H_2O_2 Treatment

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

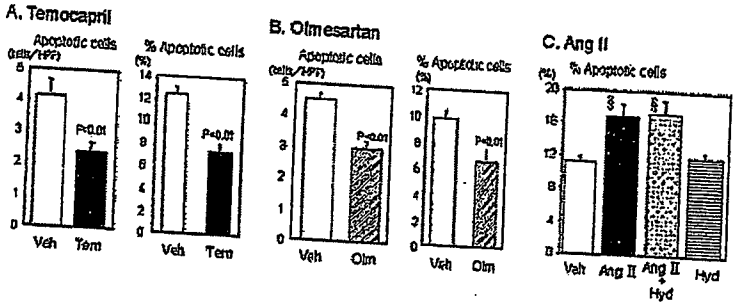

Figure 2. Effects of temocapril (A), olmesartan (B), and Ang II (C) on EC apoptosis after H_2O_2 treatment in rat carotid artery. The number of apoptotic ECs was counted per high power field (HPF; ×200), and the ratio of the apoptotic cell number to the intact cell number was calculated using *en face* specimens of the carotid artery stained with Hoechst 33342. A and B, Temocapril (Tem; 10 mg/kg per day; n=12), olmesartan (Olm; 1 mg/kg per day; n=8), or their vehicle (Veh; n=10 and n=6, respectively) was administered orally for 3 days before H_2O_2 treatment. C, Ang II (0.7 mg/kg per day) or its vehicle was administered subcutaneously for 3 days using an osmotic minipump alone (n=8 for Ang II and n=10 for vehicle) or in combination with oral administration of hydralazine (Hyd; 25 mg/kg per day; n=6 for Ang II and n=6 for vehicle; single administration for 5 days and coadministration with Ang II for 3 days) before H_2O_2 treatment. §$P<0.01$ vs vehicle. Values are expressed as mean±SEM.

Effect of RAS Inhibitors and Ang II on EC Apoptosis After H_2O_2 Treatment in Rats

The effects of an ACE inhibitor, temocapril, and an AT1 receptor blocker, olmesartan, on EC apoptosis were examined at 24 hours after H_2O_2 treatment because the peak of apoptosis was observed at 6 to 24 hours.[24] Administration of 10 mg/kg per day temocapril or 1 mg/kg per day olmesartan for 3 days before H_2O_2 treatment did not significantly change body weight, heart rate, or blood pressure, but this dose of temocapril effectively inhibited plasma ACE activity (data not shown). The number and percentage of apoptotic cells, as determined using *en face* specimens with Hoechst 33342 staining, were significantly decreased by temocapril compared with vehicle (Figure 2A; supplemental Figure I, available online at http://www.hypertensionaha.org). Olmesartan showed a comparable inhibitory effect on EC apoptosis (Figure 2B).

Ang II was administered for 3 days in combination with hydralazine to eliminate the effect of Ang II on blood pressure. Consequently, systolic blood pressure was higher in rats administered Ang II alone (161±5 mm Hg; $P<0.01$) than in the other groups of rats: 123±3 mm Hg in the vehicle group, 129±7 mm Hg in the Ang II plus hydralazine group, and 114±4 mm Hg in the hydralazine group. In contrast to RAS inhibitors, Ang II administration augmented EC apoptosis independent of the pressor effect because coadministration of hydralazine did not influence EC apoptosis (Figure 2C).

Inhibitory Effect of Temocapril on Neointimal Formation

We examined whether inhibition of EC apoptosis by temocapril would result in a reduction of neointimal formation. To do so, histological analysis of the carotid artery was performed 2 weeks after H_2O_2 treatment. Temocapril significantly decreased the neointimal area and the intima/media area ratio: intima/media area ratio was 0.18±0.02 in the vehicle group versus 0.12±0.02 in the temocapril group (n=9; $P<0.05$; supplemental Figure II). Because temocapril was administered for only 3 days before H_2O_2 treatment, it is suggested that inhibition of EC apoptosis may play a mechanistic role in attenuation of neointimal formation, although ACE inhibitors have various effects such as anti-inflammation and antimigration as well.

Effect of RAS Inhibitors on H_2O_2-Induced EC Apoptosis in Culture

To reproduce oxidative stress–induced EC apoptosis in culture, we applied 0.2 mmol/L H_2O_2 to cultured ECs derived from a bovine carotid artery for 1.5 hours based on dose- and time-response experiments. EC apoptosis, as determined by DNA fragmentation and caspase-3 activity, was induced at 24 hours after H_2O_2 treatment. Comparable to in vivo experiments, temocapril inhibited EC apoptosis in a dose-dependent manner (Figure 3A and 3B). The inhibitory effect on EC apoptosis was mimicked by 10 μmol/L olmesartan (Figure 3C), but an AT2 receptor blocker, PD123319, did not influence EC apoptosis (supplemental Figure IIIA). The involvement of NO in the effect of temocapril was examined using an NO synthase inhibitor, L-NAME, because ACE inhibitors stimulate NO production via the inhibition of bradykinin degradation.[12] However, L-NAME did not influence the effect of temocapril (supplemental Figure IIIB).

To make the interaction between H_2O_2 and Ang II clear, dose response and combined effects of both agents on EC apoptosis and 8-isoprostane formation, a marker of oxidative stress, were examined. As shown in Figures 3D and 4A, combination of Ang II and H_2O_2 dose-dependently stimulated EC apoptosis and 8-isoprostane formation. Conversely, temocapril and olmesartan restrained 8-isoprostane formation (Figure 4B) and intracellular DCF formation (Figure 4C; supplemental Figure IV) induced by H_2O_2, suggesting that endogenous Ang II also interacts with H_2O_2 to elevate oxidative stress levels.

ACE activity and the expression of AT1 receptor mRNA in cultured ECs were determined. ACE activity calibrated by the protein concentration was not changed after H_2O_2 treatment: 106±9% at 3 hours and 103±8% at 24 hours after H_2O_2 treatment compared with the values at baseline and 3 hours after vehicle treatment (100±3% and 96±13%, respectively; n=3). The relative amount of the AT1 receptor to the housekeeping gene G3PDH, as measured by real-time PCR analysis, was not significantly changed after H_2O_2 treatment: 91±2% at 1.5 hours during the treatment, 99±5% at 3 hours, and 102±4% at 6 hours after H_2O_2 treatment compared with vehicle treatment (100±6%; n=3). Considering negative regulation in vascular smooth muscle cells[27,28] together, upregulation of the AT1 receptor is not likely to occur in response to H_2O_2 treatment.

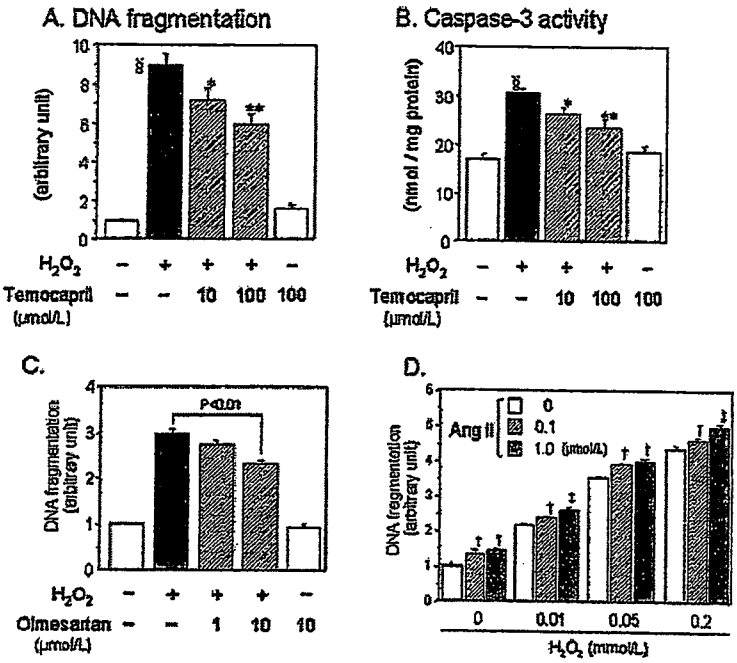

Figure 3. Effects of temocapril (A and B), olmesartan (C), and Ang II (D) on H_2O_2-induced EC apoptosis in culture. A through D, Temocapril, olmesartan, Ang II, or their vehicle was added to the culture medium 24 hours before H_2O_2 treatment until assay. EC apoptosis was evaluated 24 hours after H_2O_2 treatment (0.2 mmol/L in A through C; 0.01 to 0.2 mmol/L in D) by means of DNA fragmentation (A, C, and D; n=3) and caspase-3 activity (B; n=4). §$P<0.01$ vs H_2O_2 (−). *$P<0.05$; **$P<0.01$ vs H_2O_2 (+) + temocapril (−). †$P<0.05$ vs Ang II (−). ‡$P<0.05$ vs Ang II 0.1 μmol/L. Values are expressed as mean±SEM. Similar results were obtained in 3 independent experiments.

Discussion

This study was conducted to elucidate the role of the RAS in oxidative stress–induced EC apoptosis using a rat model and cultured ECs. Treatment with H_2O_2 did not increase ACE activity or Ang II in the rat carotid artery during the acute phase. However, administration of an ACE inhibitor, temocapril, and an AT1 receptor blocker, olmesartan, inhibited EC apoptosis in vivo. Furthermore, we demonstrated using cultured ECs that combination of Ang II and H_2O_2 dose-dependently increased EC apoptosis and 8-isoprostane formation. In addition, temocapril and olmesartan reduced but not canceled EC apoptosis and 8-isoprostane formation induced by H_2O_2, suggesting that endogenous Ang II interacts with H_2O_2 to elevate oxidative stress levels and EC apoptosis.

In vascular lesions such as atherosclerosis and intimal hyperplasia, the production of reactive oxygen species[4,5] as well as the components of the RAS[9–12] are upregulated, suggesting a possible interaction between them. A number of investigations have clarified that Ang II induces oxidative stress in vascular cells. Ang II stimulates the production of reactive oxygen species in ECs by upregulating the subunits of NAD(P)H oxidase: gp91 phox[17] and p47 phox.[18] It has been reported that the RAS enhances EC apoptosis in vitro[20,21] and contributes to endothelial dysfunction in patients with renovascular hypertension through the oxidant-dependent mechanism.[19] Conversely, it remains unknown whether oxidative stress could regulate the RAS; only 1 report has shown the modulation of ACE by oxidative stress.[29] Usui et al[29] reported that the inhibition of NO synthesis by chronic administration of L-NAME in rats augmented superoxide production and ACE activity in aortic ECs, and these effects were eliminated by treatment with

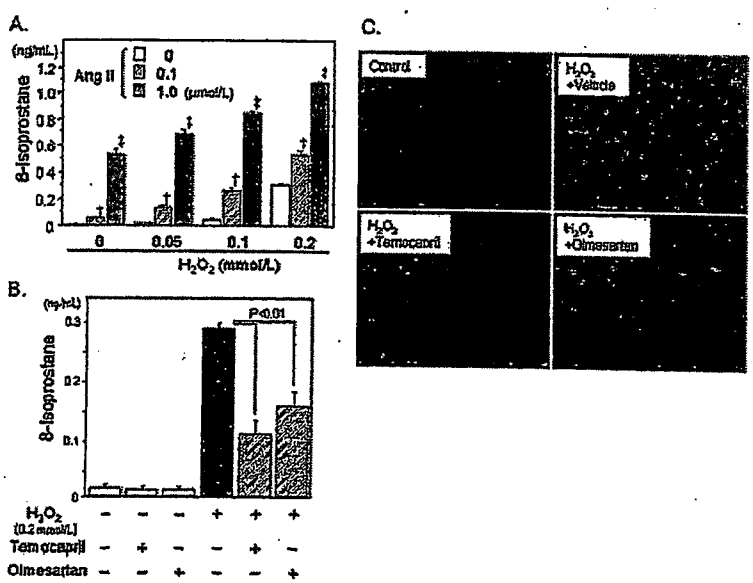

Figure 4. Effects of Ang II (A), temocapril, and olmesartan (B and C) on 8-isoprostane and DCF formation in cultured ECs. Ang II, temocapril (100 μmol/L), olmesartan (10 μmol/L), or their vehicle was added to the culture medium 24 hours before H_2O_2 treatment until assay. Then 8-isoprostane concentration in the culture supernatant and intracellular DCF intensity were measured 3 hours after H_2O_2 treatment. †$P<0.05$ vs Ang II (−). ‡$P<0.05$ vs Ang II 0.1 μmol/L. Values are expressed as mean±SEM (n=3). Similar results were obtained in 3 independent experiments.

antioxidants. In the present study, ACE activity in the carotid artery was not increased until 24 hours after H_2O_2 treatment. We also found that ACE activity was not changed after H_2O_2 treatment in cell culture experiments. Furthermore, the expression of AT1 receptor mRNA in cultured ECs, as measured using real-time PCR, was not increased after H_2O_2 treatment. Together, it is not likely that Ang II production or its receptor expression was upregulated in response to H_2O_2.

However, an ACE inhibitor, temocapril, and an AT1 receptor blocker, olmesartan, inhibited H_2O_2-induced EC apoptosis in rats as well as in cell culture experiments. No influence of L-NAME on the antiapoptotic effect of temocapril in cell culture studies indicates that the effect of temocapril was attributable to the inhibition of Ang II synthesis. An AT2 receptor blocker, PD123319, did not influence H_2O_2-induced EC apoptosis either. This result appears to be inconsistent with the previous finding[30] but suggests a minimal contribution of the AT2 receptor in H_2O_2-induced EC apoptosis or minimal expression of the AT2 receptor in the cultured ECs used in the present study. Reduction in 8-isoprostane formation by temocapril and olmesartan suggests that endogenous Ang II adds to the oxidative stress levels on top of exogenous H_2O_2; otherwise temocapril and olmesartan would have antioxidant effects independent of Ang II through currently unknown mechanisms, although the in vivo role of bradykinin/NO in the effect of ACE inhibitors and that of the AT2 receptor remain to be addressed.

Administration of Ang II provided evidence that Ang II can interact with H_2O_2 to elevate oxidative stress levels and induce EC apoptosis. In rat experiments, a high and pressor dose of Ang II was used in combination with hydralazine[31] because 3-day administration of lower doses of Ang II (0.1 to 0.2 mg/kg per day) did not show significant effects on EC apoptosis (data not shown). The cell culture experiments to examine the effect of submaximal doses of Ang II and H_2O_2 on apoptosis and 8-isoprostane formation gave us clear information that AT1 receptor signaling augments EC apoptosis by an interaction with oxidative stress. Although the doses of H_2O_2 and the time duration of exposure were optimized on the basis of the time- and dose-response experiments, the conditions in cell culture studies were different from those in animal studies. However, it has been reported that cigarette smoke, oxidized lipoproteins, and polymorphonuclear leukocytes, which play important roles in atherogenesis, can generate H_2O_2 concentrations of 0.05 to 0.2 mmol/L in vitro.[32] These reports suggest that the dosages of H_2O_2 used in the present study do not far exceed the physiological range, although direct comparison of physiological or pathophysiological conditions with those in our experiments may be inappropriate.

Considering the stimulatory effect of Ang II on free radical production,[17–19] our finding that endogenous Ang II exacerbates EC apoptosis induced by exogenous H_2O_2 is not surprising. In fact, a number of reports have shown experimentally that RAS inhibitors can reduce the production of reactive oxygen species in pathological conditions such as peripheral arteries in rats with chronic heart failure,[33] rat diabetic nephropathy,[34] and kidney mitochondria in aged rats.[35] In the clinical setting, it is reported that administration of an AT1 receptor blocker (losartan) to patients with chronic renal disease reduced urinary excretion of oxidized albumin and malondialdehyde.[36] Also, 4-week treatment with losartan or an ACE inhibitor (ramipril) in patients with coronary artery disease diminished the response of endothelium-dependent vasodilation to intracoronary administration of antioxidant vitamin C in parallel with improvement of basal endothelium-dependent vasodilation,[37] indicating that RAS inhibitors can improve endothelial function in association with a reduction of oxidative stress. In the present study, we investigated EC apoptosis, an important process that leads to endothelial dysfunction and atherosclerosis[22,23] using an in vivo model. Moreover, our finding that RAS inhibitors attenuated EC apoptosis suggests broad end-organ protective effects of RAS inhibitors, which have been used for the treatment of hypertension and heart failure.

Perspectives

We found using an in vivo model and cultured ECs that Ang II elevated oxidative stress levels and increased EC apoptosis, whereas RAS inhibitors restrained them. These findings will add new information for cardiovascular research and the clinical application of RAS inhibitors.

Acknowledgments

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Amelioration of Vascular Endothelial Dysfunction in Obstructive Sleep Apnea Syndrome by Nasal Continuous Positive Airway Pressure

— Possible Involvement of Nitric Oxide and Asymmetric NG, NG-Dimethylarginine —

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Background Asymmetric NG,NG-dimethylarginine (ADMA) is an endogenous inhibitor of endothelial nitric oxide (NO) synthase and its plasma concentration is elevated in patients with cardiovascular risk factors, including hyperlipidemia, hypertension, diabetes, and hyperhomocysteinemia. Obstructive sleep apnea syndrome (OSAS) has been attracting attention as a risk factor for cardiovascular disorders because it often accompanies hypertension, obesity, glucose impairment, and dyslipidemia, all of which are factors in metabolic syndrome and risk factors for cardiovascular disease.

Methods and Results In the present study, flow-mediated vasodilatation (FMD) of the brachial artery and plasma concentrations of ADMA were measured before and after nasal continuous positive airway pressure (nCPAP) therapy, which abrogates apnea, in 10 male patients aged 36–69 years old, who were given a diagnosis of OSAS by polysomnography. The percent FMD (%FMD) improved significantly from 3.3±0.3% to 5.8±0.4% ($p<0.01$) and 6.6±0.3% ($p<0.01$), before, 1 week, and 4 weeks after nCPAP, respectively. At the same time, the plasma NOx concentrations, metabolites of NO, tended to increase, but the plasma ADMA concentration decreased inversely to %FMD and NOx. A negative correlation between %FMD and plasma ADMA concentration, and a positive correlation between %FMD and plasma NOx concentrations were observed.

Conclusion Nasal CPAP improves endothelial function, in part by the decreasing ADMA concentration, thereby potentiating NO production. (*Circ J* 2005; **69:** 221–226)

Key Words: Asymmetric NG, NG-dimethylarginine (ADMA); Flow-mediated dilatation; Nasal continuous positive airway pressure; Obstructive sleep apnea syndrome

Endothelial dysfunction is recognized as an early phase of arteriosclerosis[1] and an important cause of that dysfunction is impaired nitric oxide (NO) release from the endothelium. Endothelial NO is a key regulator of vascular homeostasis; it induces vasorelaxation by generating cyclic GMP in the underlying smooth muscle cells, and prevents monocyte adhesion to the endothelium, platelet activation, and smooth muscle cell proliferation. Hence, impaired NO release from injured endothelial cells is regarded as an initiator and promoter of arteriosclerosis.

Endothelial NO is produced when L-arginine is converted to L-citrulline by the enzyme endothelial nitric oxide synthase (eNOS). Endothelial NOS is inhibited by endogenous inhibitors, NG-monomethyl-L-arginine (L-NMMA) and asymmetric dimethylarginine (ADMA), which are structural analogues of L-arginine?[2,3] Plasma ADMA is eliminated by renal excretion and by degradation to citrulline and dimethylamine by the enzyme dimethylarginine dimethylaminohydrolase (DDAH)[4] Increased plasma concentration of ADMA is associated with hypertension[5] pulmonary hypertension[6] hypercholesterolemia[7,8] carotid intima–media thickening[9] severe peripheral artery occlusive disease,[10] and the clustering of coronary risk factors[9] These findings suggest that ADMA is responsible for endothelial dysfunction.

Obstructive sleep apnea syndrome (OSAS) has been recently attracting attention as a significant disorder. Frequent apnea/hypopnea attacks followed by arousal results in insufficient sleep at night, causing daytime sleepiness, leading to work inefficiency, and even traffic accidents. In addition, OSAS often accompanies hypertension, obesity, glucose intolerance, and dyslipidemia, all of which are factors in metabolic syndrome. Hence, OSAS is recognized as a risk factor for cardiovascular disease![11–14] It has been

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Table 1 Clinical Characteristics of the Study Subjects With Obstructive Sleep Apnea Syndrome

	Before nCPAP	*1 week after nCPAP*	*4 weeks after nCPAP*
Body mass index (kg/m²)	*29.1±4.8*	*28.8±4.6*	*28.0±4.7*
Systolic BP (mmHg)	*131±15.2*	*128±10.3*	*133±8.8*
Diastolic BP (mmHg)	*79±9.3*	*75±6.2*	*81±6.5*
Heart rate (beats/min)	*74±14*	*72±10*	
Creatinine (mg/dl)	*0.85±0.21*		*0.83±0.22*
Uric acid (μmol/L)	*387±59*	*387±65*	*363±48*
Total cholesterol (mmol/L)	*5.23±0.72*	*4.79±0.61*	*4.71±0.62*
HDL-cholesterol (mmol/L)	*1.06±0.20*	*1.04±0.19*	*1.19±0.26*
Triglyceride (mmol/L)	*1.66±0.55*	*1.60±0.75*	*1.28±0.39*
LDL-cholesterol (mmol/L)	*3.39±0.72*	*3.03±0.69*	*2.92±0.72*
Fasting plasama glucose (mmol/L)	*5.83±1.28*	*5.72±1.40*	*5.77±1.00*
HemoglobinA1c (%)	*5.9±1.2*	*5.9±1.4*	*5.5±1.0*
Apnea/hypopnea index	*33±14.7*	*3.9±3.7*	
	p<0.01		
Desaturation index	*11.1±8.2*	*0.26±0.43*	
	p<0.01		

All values are presented as mean±SEM. Other than the apnea/hypopnea index and desaturaion index, none were statistically significant before or after nCPAP (1 week and 4 weeks). BP, blood pressure; desaturation index, duration of SpO2 <90%/total sleep time (%); HDL, high-density lipoprotein; LDL, low-density lipoprotein; nCPAP, nasal continuous positive airway pressure.

also reported that endothelium-dependent vasodilatation is impaired in OSAS patients![15,16] Currently, the most effective therapy for OSAS is nasal continuous positive airway pressure (nCPAP), which eliminates the upper airway obstruction.

In the present study, we examined the impact of nCPAP on endothelial function by measuring the flow-mediated vasodilatation (FMD) of the brachial artery before and after nCPAP. We also examined the temporal change in both the NO metabolites (NOx) and ADMA to understand the mechanism of improvement in FMD by nCPAP.

Methods

Patients

This study was performed in 10 men, aged 36–69 years (53.3±10.5, mean±SD), who were admitted to the Department of Geriatric Medicine, The University of Tokyo Hospital, given a diagnosis of OSAS by polysomnography and then treated with nCPAP (Table 1). Seven patients had hypertension, 5 of whom were on medication; 4 patients were diabetic, one of whom was on oral medication. The pressure for the nCPAP was adjusted by titration with auto-CPAP followed by manual titration. Measurements of FMD, plasma ADMA, and NOx were performed before, 1, and 4 weeks after nCPAP. The diagnostic criterion for OSAS was either an apnea/hypopnea index (AHI) >10 or SpO2 <90% for more than 5 min or more than 1% of total sleep time![17] The indication for nCPAP was (1) AHI >20 or (2) SpO2 <90% more than 20 min or more than 5% of total sleep time. The study protocol was approved by the ethics committee of The University of Tokyo Hospital, and written informed consent was obtained from each patient.

Measurement of Flow-Mediated Vasodilatation

Percent flow-mediated, endothelium-dependent, vasodilatation (%FMD) and percent nitroglycerine-mediated, endothelium-independent, vasodilatation (%NTG) were determined by ultrasound![18,19] The subjects rested quietly for 15–20 min on a bed in a temperature-controlled room. The blood flow and vessel diameter of the upper right brachial artery were measured using a 7.5-MHz ultrasound linear array transducer. Forearm ischemia was induced by a blood pressure Manschette tourniquet set at 250 mmHg for 5 min and subsequent rapid deflation of the tourniquet resulted in FMD. The change in diameter caused by FMD was expressed as the percent change relative to that of the initial resting scan. After the measurement of FMD, the subjects rested quietly for 15 min and complete recovery of the vessel diameter was confirmed. A sublingual spray of NTG (Myocor spray; 0.3 mg/spray) was administered, and the flow rate and vessel diameter of the same vessel were determined 3–5 min later. The diameter of the upper brachial artery was determined by taking the mean values determined from 4 images. Changes in diameter of 0.1–0.2 mm can be detected accurately with this method![20,21] The coefficient of variation for the measurements of FMD was 5.84±0.25% and that for NTG-induced dilation was 3.97±0.24%, as reported previously![20,21] The coefficient of variation for reproducibility of this ultrasound determination of FMD was 9.77±0.82% and that of NTG-induced dilation was 7.24±0.49%.

Measurement of ADMA

The plasma concentration of ADMA was measured by high-performance liquid chromatography (HPLC) with a fluorescent detection method![22] using blood samples that had been collected in EDTA tubes. The plasma was separated and 10 μl was added to 40 μl of mobile phase solution for HPLC. The arginine analogues were adsorbed in the positive ion-exchange column, after which the column was switched and the sample was injected into a separation column. NMMA, ADMA, and symmetric dimethylarginine (SDMA) were separated by ion-pair chromatography and then detected by adding fluorescent derivatization reagent, O-phthalaldehyde and thiol. The NMMA, ADMA, and SDMA calibration curves straightened over the range of 0.05–5.0 μmol/L and their respective detection limits were 0.005 μmol/L, 0.008 μmol/L, and 0.01 μmol/L (S/N=2). The intra-day variations for NMMA, ADMA, and SDMA were 4.6, 4.3, and 6.4%, respectively, and 6.1, 5.8, and 7.0%, for the inter-day variations. The HPLC solid and mobile phases were as follows: positive ion-exchange column: Capcell Pak MF-SCX (10×40 mm, inner diameter, 5 μm,

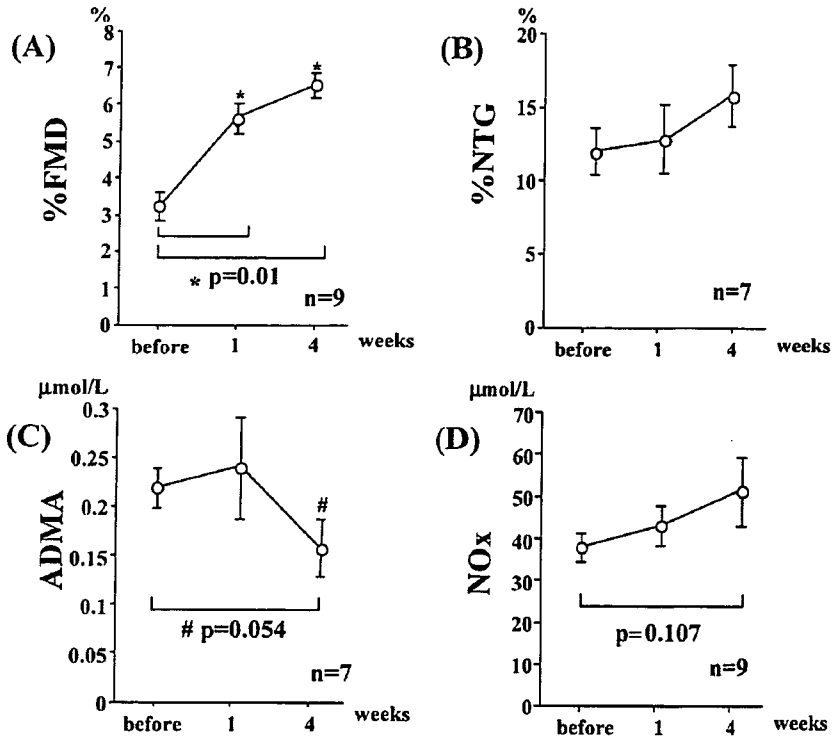

Fig 1. Changes in %FMD (A), %NTG (B), plasma ADMA concentrations (C), and plasma NOx concentrations (D) before and after nCPAP in OSAS patients. All measurements were performed before, 1 and 4 weeks after nCPAP. All values are presented as mean±SEM.

Shiseido, Tokyo); separation column: Capcell Pak MG-C18 (250×4.6 mm, inner diameter, 5 μm, Shiseido); mobile phase: 70 mmol/L sodium phosphate buffer (pH 6.7) containing 15 mmol/L cyclohexanecarboxylate and 1.5 mmol/L octanoate; flow rate: 1.0 ml/min; derivatization reagent solution: 3.7 mmol/L 2-mercaptopropionic acid in 0.2 mol/L borate buffer (pH 9.8) 3.0 mmol/L O-phthaldehyde in 0.2 mol/L borate buffer (pH 9.8); flow rate: 0.3 ml/min. The elute was monitored at 450 nm with excitation set at 337 nm.

Measurement of Nitrate and Nitrite (NOx)

NOx was measured at SRL Co, Ltd, Tokyo, by the Griess method. The samples were deproteinated and separated into nitrates and nitrites. After all the nitrates were reduced to nitrites, the samples were reacted with naphthylethylamine, and the product was determined by absorbance at 540 nm.

Statistics

All values are presented as the mean±SEM. The data were analyzed by one-factor ANOVA and the Student Newman-Keuls test was performed to test the significance of the differences. Statistical significance was made when $p<0.05$.

Results

All patients were given a diagnosis of OSAS and were indicated for nCPAP treatment, which remarkably improved both the apnea/hypopnea index and desaturation index (Table 1). Neither renal function nor the factors related to metabolic syndrome changed significantly after nCPAP (Table 1), indicating that short-term treatment does not improve patients' metabolic status. However, the %FMD changed significantly from 3.3±0.3% before nCPAP to

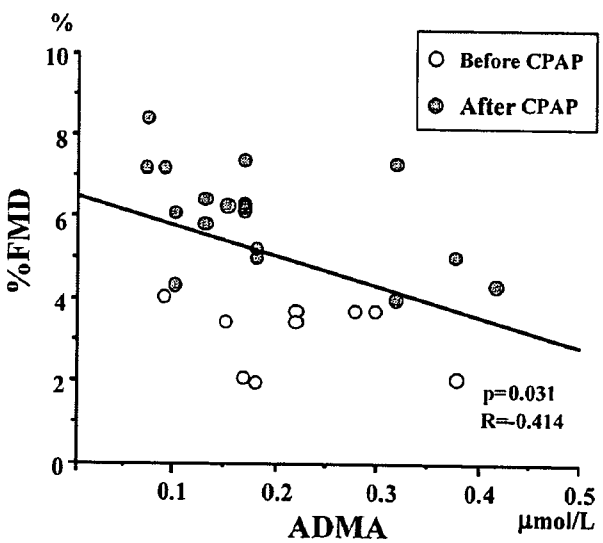

Fig 2. Correlation between %FMD and plasma ADMA concentrations before and after nCPAP. Black circles indicate values before nCPAP and white circles indicate values after nCPAP.

5.8±0.4% ($p<0.01$) at 1 week, and 6.6±0.3% ($p<0.01$) 4 weeks after nCPAP (Fig 1A). We confirmed that the basal diameter of the brachial artery was the same before and after nCPAP. No significant change was observed in %NTG, suggesting that nCPAP does not affect endothelium-independent vasodilatation (Fig 1B). The plasma concentrations of ADMA, the endogenous inhibitor of eNOS, decreased inversely to the improvement of %FMD at 4 weeks after nCPAP: 0.22±0.27 μmol/L before nCPAP, 0.21±0.44 μmol/L at 1 week, and 0.16±0.27 μmol/L at 4 weeks after nCPAP ($p=0.054$ by a paired 2 group test) (Fig 1C).

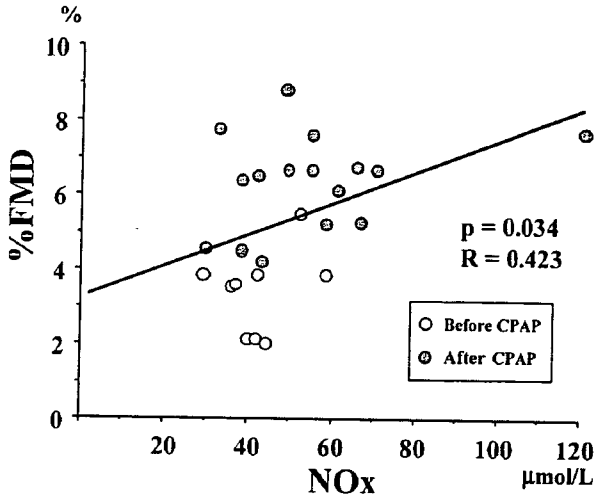

Fig 3. Correlation between %FMD and plasma NOx concentrations before and after nCPAP. Black circles indicate values before nCPAP and white circles indicate values after nCPAP.

Plasma NOx concentrations increased slightly, though not significantly, in parallel with the improvement of %FMD: 37.9±2.8 μmol/L before nCPAP, 43.0±4.6 μmol/L at 1 week after nCPAP, and 51.4±8.3 μmol/L at 4 weeks after nCPAP (Fig 1D).

Although neither the decrease in plasma ADMA concentration nor the increase in plasma NOx concentration was statistically significant, the results imply that the decreased plasma ADMA concentration lead to the improvement in %FMD by increasing NO production in the vessel wall. In fact, a negative correlation was found between %FMD and plasma ADMA concentration when all values (before, 1 week, and 4 weeks after nCPAP) were analyzed (Fig 2). Although the plasma NOx concentrations did not change significantly after nCPAP, a positive correlation was found between them and %FMD when all values (before, 1 week, and 4 weeks after nCPAP) were analyzed (Fig 3).

Discussion

Patients with cardiovascular risk factors show diminished endothelium-dependent vasodilatation[23] and elevated plasma ADMA concentrations[9] the latter being also increased in patients with vasospastic angina;[24] and in those with hypercholesterolemia, hypertriglyceridemia and diabetes mellitus, concomitant with impaired endothelium-dependent vasodilatation.[7,25,26] Endothelium-dependent vasodilatation has also been found to be impaired in patients with OSAS;[5,27–29] but their plasma ADMA concentrations were not determined in those studies. In the present study, endothelium-dependent vasodilatation was ameliorated with the improvement of apnea by nCPAP. Concomitantly, the improvement in endothelium-dependent vasodilatation paralleled negatively the plasma ADMA concentration and positively paralleled the plasma NOx concentration. The decrease in ADMA did not appear to be mediated by improved renal function or sympathetic nerve activity, known regulators of plasma ADMA, according to our data for serum creatinine and heart rate (Table 1). Our findings suggest that nCPAP reduces the concentration of ADMA, thereby enhancing NO production and leading to an improvement of endothelium-dependent vasodilatation.

One of the most deleterious features of OSAS is nocturnal hypoxia. It has been reported that serum NOx concentrations decline and there is a negative correlation between serum NOx concentration and the severity of OSAS.[30] We did not find such a negative correlation in the present study (data not shown). However, with respect to ADMA, we found a negative correlation between the difference in the plasma ADMA concentrations before and after nCPAP (ΔADMA) and the desaturation index (DI) before nCPAP, and ΔADMA and the difference of DI before and after nCPAP (ΔDI) (data not shown). The ΔADMA did not correlate with ΔAHI, which indicates that the plasma ADMA concentration parallels the severity of OSAS with regard to desaturation, but to apnea.

With respect to hypoxia and ADMA, it is reported that the expression of the dimethylarginine dimethylaminohydrolases (DDAH), the enzymes that catalyze the degradation of ADMA, is decreased in hypoxia-induced pulmonary hypertension;[6,31] but it is not known whether DDAHs are directly downregulated by hypoxia or upregulated by oxygenation, leading to the reduction of ADMA. Another plausible mechanism for impaired endothelium-dependent vasodilatation in OSAS is the reduction in eNOS protein, based on the finding that eNOS protein was decreased in the rat aorta under low oxygen tension;[32] and that hypoxia decreases the expression of eNOS mRNA and protein in cultured endothelial cells.[33] In addition, the present result that %FMD increased but plasma ADMA and NOx concentrations were unchanged at 1 week after nCPAP (Fig 1A,C, and D) suggests that FMD starts to improve before ADMA and NOx start to change. This indicates that FMD is regulated in part by the ADMA–NO axis.

There are several studies showing that OSAS is a risk factor for cardiovascular disease, but only a few have reported the prognosis of OSAS. He et al showed that in 385 untreated patients, the 9-year survival rate was significantly lower in those with apnea index (AI) >20 compared with those with an AI <20.[11] Noda et al showed that the survival rate is low in OSAS patients depending on their age; the prognosis in the middle-aged OSAS patients depends on the complication of hypertension and severity of the oxygen desaturation, but not on AHI.[34] OSAS patients suffer from a high incidence of cardiovascular and cerebrovascular diseases;[2–14] and the mortality rate increases with the severity of OSAS;[11,14] with the main causes of death being ischemic heart disease and cerebrovascular disease. Compared with the general population, OSAS patients have a 2-fold greater incidence of hypertension, 2–3-fold incidence of ischemic heart disease, and 3–5-fold incidence of cerebrovascular disease;[35] and the mortality rate from total vascular events is 2.7-fold greater than that of non-OSAS patients. In addition, it was recently reported that sleep apnea is common in patients with idiopathic cardiomyopathy.[36]

Hypertension is a frequent complication of OSAS. It has been reported that the mean blood pressure increases with the severity of the sleep apnea.[37] Hypertension is significantly associated with a lower survival rate in the middle-aged population.[34] Hypertension can be induced in rats by putting them in hypoxic conditions for 8 h daily;[38] and in dogs by repetitive airway occlusion while sleeping.[39] Although the precise mechanism of this induction is not understood, augmented sympathetic nerve tension following repetitive hypoxia is thought to be one of the causes for the rise in blood pressure. Indeed, it is reported that urinary

catecholamine excretion during the night is increased in OSAS patients. Augmented sympathetic nerve activity during the night may also elevate vessel tone, leading to elevated blood pressure not only at night, but also during the day. Furthermore, the serum NOx concentration negatively parallels blood pressure in OSAS patients,[30] indicating that NO production from endothelial cells plays a significant role in the elevation of blood pressure. With respect to the effect of nCPAP on blood pressure, it has been shown that blood pressure falls after adequate treatment with nCPAP,[40,41] and the assumed mechanism is reduced peripheral vascular resistance.[42] As well, nCPAP changes the diurnal pattern of blood pressure from non-dipper to dipper, which presumably reduces the risk of coronary events.[43] In the present study, we did not find any decline in systolic or diastolic pressure over the 4 weeks and it may take longer before the effect on blood pressure becomes apparent.

The importance of vascular endothelial cells in OSAS has been shown in relation to vascular events.[30,44] OSAS patients are exposed to hypoxia for prolonged periods, which would damage endothelial cells, evidenced by the enhanced release of thrombomodulin and von Willebrand factor (vWF). Plasma vWF concentrations, which are high in OSAS patients compared with control subjects, were reduced significantly at 1 month after nCPAP, which is in agreement with our study result that endothelial dysfunction is improved by nCPAP.

Study Limitations

We were unable to find unequivocal causal relationships of ADMA, NOx, and FMD; a negative correlation between ADMA and FMD, and a positive correlation between NOx and FMD were discovered when all values (before, 1 week, and 4 weeks after nCPAP) were analyzed, although no correlation was found when the values were analyzed separately. This result is related to the limited number of subjects and in this sense, the occurrence of type I and type II errors is the default of the current study. Another limitation of the present study is the lack of an age-matched, and BMI-matched control group because of the very high BMI of the study subjects in relation to the rest of the Japanese population. We previously showed that %FMD declines significantly in subjects with one or more coronary risk factors from 6.7±0.3% (control group; 56.8±1.0 years old) to 4.8±0.5%.[23] The ages of those subjects were close to those of the patients in the current study. From these data, the %FMD of 3.3±0.3% before nCPAP in OSAS patients is considered very low compared with the %FMD of healthy subjects in our previous study. From this viewpoint, the endothelial function appears to be impaired in the present OSAS patients and we need to set up a more precise control group to clarify this.

Conclusion

We found that nCPAP improves endothelial function (FMD), in part by reducing ADMA, thereby increasing L-arginine availability and NO production. In this regard, nCPAP is effective for treating vascular dysfunction as well as sleep disorders. OSAS is now recognized as a vicious cluster of sleep, metabolic, and vascular disorders and we propose that measuring FMD, ADMA, and NOx is useful for evaluating the vascular conditions.

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