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ORIGINAL ARTICLE
Ischemic Heart Disease

Prognostic Effects of Calcium Channel Blockers in Patients With Vasospastic Angina

– A Meta-Analysis –

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Background: Although calcium channel blockers (CCB) are highly effective for suppression of vasospastic angina (VSA) attacks, their prognostic effects in VSA patients remain to be examined in a large number of patients.

Methods and Results: Databases for related papers were searched and then a meta-analysis regarding the effects of CCB on major adverse cardiovascular events (MACE) in Japanese VSA patients with the 4 previous studies was performed. A total of 1,997 patients with positive coronary spasm provocation tests were evaluated. They were treated with either alone or combination of benidipine (n=320), amlodipine (n=308), nifedipine (n=182) or diltiazem (n=960). MACE were observed in 143 patients (cardiac death: 36, myocardial infarction: 51, heart failure: 26, stroke: 65, and aortic aneurysm: 11). The hazard ratio for the occurrence of MACE was significantly lower in patients treated with benidipine than in those with diltiazem. There was no significant difference in the clinical characteristics affecting the occurrence of MACE among the 4 CCB groups. Furthermore, the hazard ratio for the occurrence of MACE was significantly lower in those treated with benidipine, even after correction for patient characteristics that could have affected the occurrence of MACE (hazard ratio 0.41, P=0.016).

Conclusions: These results suggest that among the 4 major CCB that effectively suppress VSA attacks in general, benidipine showed significantly more beneficial prognostic effects than others. (*Circ J* 2010; **74:** 1943–1950)

Key Words: Calcium channel blockers; Prognosis; Vasospastic angina

The prevalence of coronary vasospasm is high in Japanese patients with ischemic heart disease[1,2] and acute myocardial infarction (AMI).[3] Therefore, the control of vasospastic angina (VSA) has been an important clinical issue in Japan.[4–7] Calcium channel blockers (CCB) have been widely used to suppress VSA attacks and to improve the outcome of VSA patients.[4,8,9] However, comparison of the prognostic effects of CCB in VSA patients remains to be performed in a large number of patients. During the past decades, several observational studies showed that the outcome of VSA patients vary, depending on the CCB used.[10–14] In the 1980s, it was shown that 80–90% of VSA patients were treated with CCB and the major adverse cardiovascular events (MACE) rate was relatively low during the 5-year follow-up.[8] Recently, the usage of CCB has further increased to 90–95% unless otherwise contraindicated. Thus, in order to compare the actual difference among the major CCB, it is necessary to perform a large-scale clinical trial. Another possible approach is to perform a meta-analysis, utilizing the database created from previously published papers that compared the prognostic effects of CCB. In the present study, we thus performed a meta-analysis in which we compared the prognostic effects of CCB in Japanese patients with VSA.

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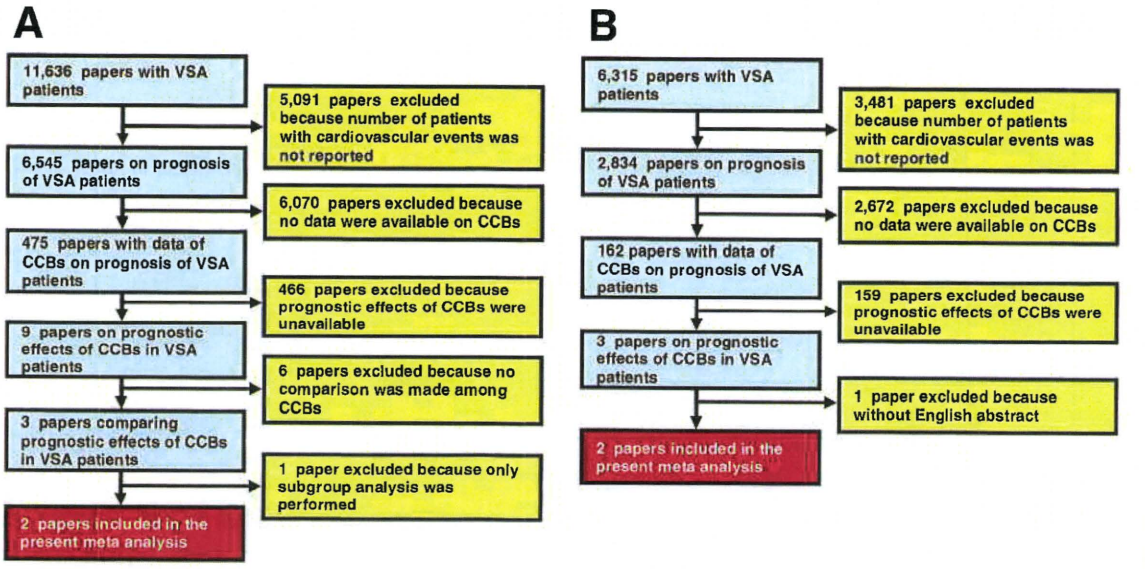

Figure 1. Summary of identification and selection of previous papers. According to the keywords, papers were systemically searched from (**A**) the database of MEDLINE® for English articles (from 1950 to 2008), and (**B**) from the database of Ichushi® (Japana centra revuo medicina) for Japanese articles with an English abstract (from 1983 to 2008). VSA, vasospastic angina; CCB, calcium channel blockers.

Table 1. Number of Patients, Spasm Provocation Test and Treatment With Calcium Channel Blockers in the 4 Studies Included in the Present Meta-Analysis

Study (years)	Total number of patients (n)	Follow-up rate (%)	Spasm provocation test	Calcium channel blockers			
			Positive response* (n)	Benidipine (n, %)	Amlodipine (n, %)	Nifedipine (n, %)	Diltiazem (n, %)
Ito (2004)[10]	726	92	665	148 (22)	111 (17)	106 (16)	405 (61)
Sueda (2006)[12]	194	83	161	52 (32)	35 (22)	13 (8)	46 (29)
Io (2007)[11]	1,146	91	879	42 (5)	149 (17)	39 (4)	358 (41)
Kodama (2007)[13]	389	75	292	78 (27)	13 (4)	24 (8)	151 (52)
All studies	2,455	88	1,997	320 (16)	308 (15)	182 (9)	960 (48)

*Considered positive when intracoronary administration of acetylcholine or ergonovine induced an ischemic ECG change or chest pain with 75% or greater constriction of the coronary artery.

Methods

Study Eligibility Criteria and Search Strategy

We searched the database of MEDLINE® for papers published in English from 1950 to December, 2008 and the Japana Centra Revuo Medicina (Ichushi®) for papers published in Japanese with an English abstract from 1983 to December, 2008. The keywords used were 16 CCB (nifedipine, nicardipine, nisoldipine, nitrendipine, nilvadipine, manidipine, benidipine, amlodipine, barnidipine, efonidipine, felodipine, clinidipine, aranidipine, azelnidipine, diltiazem, or verapamil), prognosis, and VSA and related terms (vasospasm, coronary VSA, rest angina, variant angina). The papers in Japanese were restricted to those with an English abstract. Among the 11,636 papers in English searched by the database of MEDLINE®, 2 papers were finally selected, in which the effects of CCB on the occurrence of MACE in VSA patients were compared (**Figure 1A**).[10,11] In addition, 2 papers in Japanese were selected[12,13] among the 6,315 papers searched by the database of Ichushi® (**Figure 1B**). Permission to perform a meta-analysis was obtained from the authors of the selected 4 papers.[10–13] We asked them to provide unlinkable, anonymous raw data (database), which we used to create a new database. This enabled us to analyze the data from 1,997 VSA patients with positive response to coronary spasm provocation tests. The coronary spasm provocation test was considered positive when an ischemic ECG changes and/or chest pain with coronary constriction was induced by intracoronary administration of acetylcholine or ergonovine, based on the 2008 Guidelines of the Japanese Circulation Society.[15] In the present study, the baseline point was defined as the day of discharge after the diagnosis of VSA, and the follow-up period was redefined from the 4 papers.

Comparison of CCB Treatments

Among the 1,997 VSA patients, 1,554 (77.8%) were treated with CCB, including benidipine (n=320), amlodipine (n=308), nifedipine (n=182) and diltiazem (n=960) (**Table 1**). Among

Table 2. Clinical Characteristics of Patients With Vasospastic Angina Treated With Each Calcium Channel Blocker

	Benidipine (n=320) (n, %)	Amlodipine (n=308) (n, %)	Nifedipine (n=182) (n, %)	Diltiazem (n=960) (n, %)	P value
Age					
Years (mean±SD)	64.6±9.8	64.4±9.5	63.4±9.9	62.6±10.3	0.015
Median (min-max)	65.4 (37–91)	65.0 (33–91)	63.0 (32–85)	62.6 (32–92)	
Sex					
Male	196 (61)	216 (70)	134 (74)	683 (71)	0.005
Female	124 (39)	92 (30)	48 (26)	277 (29)	
BMI, kg/m² (mean±SD)	23.9±3.4	23.8±3.0	23.4±2.9	23.5±3.0	0.173
Smoking	135 (42)	125 (41)	64 (35)	388 (40)	0.471
Family history of IHD	41 (13)	35 (11)	29 (16)	137 (14)	0.904
Hypertension	165 (52)	178 (58)	119 (65)	362 (38)	<0.001
Diabetes mellitus	61 (19)	46 (15)	29 (16)	141 (15)	0.343
Hyperlipidemia	150 (47)	123 (40)	58 (32)	316 (33)	<0.001
Previous MI	24 (8)	24 (8)	26 (14)	93 (10)	0.049
LVEF, % (mean±SD)	71.0±10.8	68.6±12.5	69.9±11.8	70.1±10.4	0.506
Coronary artery disease	56 (18)	56 (18)	32 (18)	163 (17)	0.952
0 VD	253 (79)	242 (79)	136 (75)	762 (79)	0.807
1 VD	39 (12)	46 (15)	24 (13)	124 (13)	
2 VD	16 (5)	9 (3)	6 (3)	32 (3)	
3 VD	1 (0)	1 (0)	2 (1)	7 (1)	
Treatment					
Nitrates	143 (45)	167 (54)	111 (61)	574 (60)	<0.001
Nicorandil	71 (22)	53 (17)	47 (26)	164 (17)	0.015
Aspirin	48 (15)	44 (14)	17 (9)	131 (14)	0.594
Statins	33 (10)	32 (10)	15 (8)	86 (9)	0.598
ARB	17 (5)	30 (10)	8 (4)	36 (4)	0.004
ACE inhibitors	33 (10)	36 (12)	15 (8)	51 (5)	0.001
β-blockers	34 (11)	33 (11)	14 (8)	67 (7)	0.076

BMI, body mass index; IHD, ischemic heart disease; MI, myocardial infarction; LVEF, left ventricular ejection fraction; VD, vessel disease; ARB, angiotensin II receptor blockers; ACE, angiotensin converting enzyme.

Table 3. The Number of Each Event Observed in Patients With Vasospastic Angina Treated With Either of 4 Calcium Channel Blockers

	Benidipine (n=320) (n, %)	Amlodipine (n=308) (n, %)	Nifedipine (n=182) (n, %)	Diltiazem (n=960) (n, %)	P value*
Overall (n=1,997)					
MACE	8 (2.5)	17 (5.5)	19 (10.4)**,‡	83 (8.6)‡	<0.001
Cardiac death	1 (0.3)	4 (1.3)	8 (4.4)**,‡	21 (2.2)†	<0.001
Myocardial infarction	4 (1.3)	4 (1.3)	5 (2.7)	31 (3.2)	0.109
Stroke	5 (1.6)	9 (2.9)	6 (3.3)	38 (4.0)†	0.213
Heart failure	4 (1.3)	6 (1.9)	3 (1.6)	17 (1.8)	0.911
Aortic aneurysm	2 (0.6)	2 (0.6)	2 (1.1)	7 (0.7)	0.937
Total death	10 (3.1)	15 (4.9)	16 (8.8)‖	65 (6.8)†	0.030

MACE, major adverse cardiovascular events.
*χ^2-test (among 4 groups) **P<0.05 (vs amlodipine), †P<0.05, ‖P<0.01, ‡P<0.001 (vs benidipine).

those treated with CCB, 1,349 patients were treated with a single CCB, including benidipine (n=219), amlodipine (n=199), nifedipine (n=143) and diltiazem (n=788). As the number of VSA patients treated with other CCB was small, these patients were excluded for comparison of the 4 CCB, although they were included in the whole meta-analysis. For patient characteristics, we examined the baseline data on age, gender, body mass index (BMI), smoking, family history of ischemic heart disease, risk factors (hypertension, diabetes mellitus, and hyperlipidemia), previous myocardial infarction (MI), left ventricular ejection fraction (LVEF), and the presence of significant coronary artery disease (CAD, 75% or greater coronary stenosis on coronary angiography). Co-treatment with other class of drugs also was examined, including nitrate preparations, nicorandil, statins, angiotensin II receptor blockers (ARB), angiotensin converting enzyme (ACE) inhibitors and β-blockers. The data for age, BMI and LVEF were handled by the use of categorization (age, 10-year intervals; BMI, ≥22.5 kg/m²; LVEF, <45%).

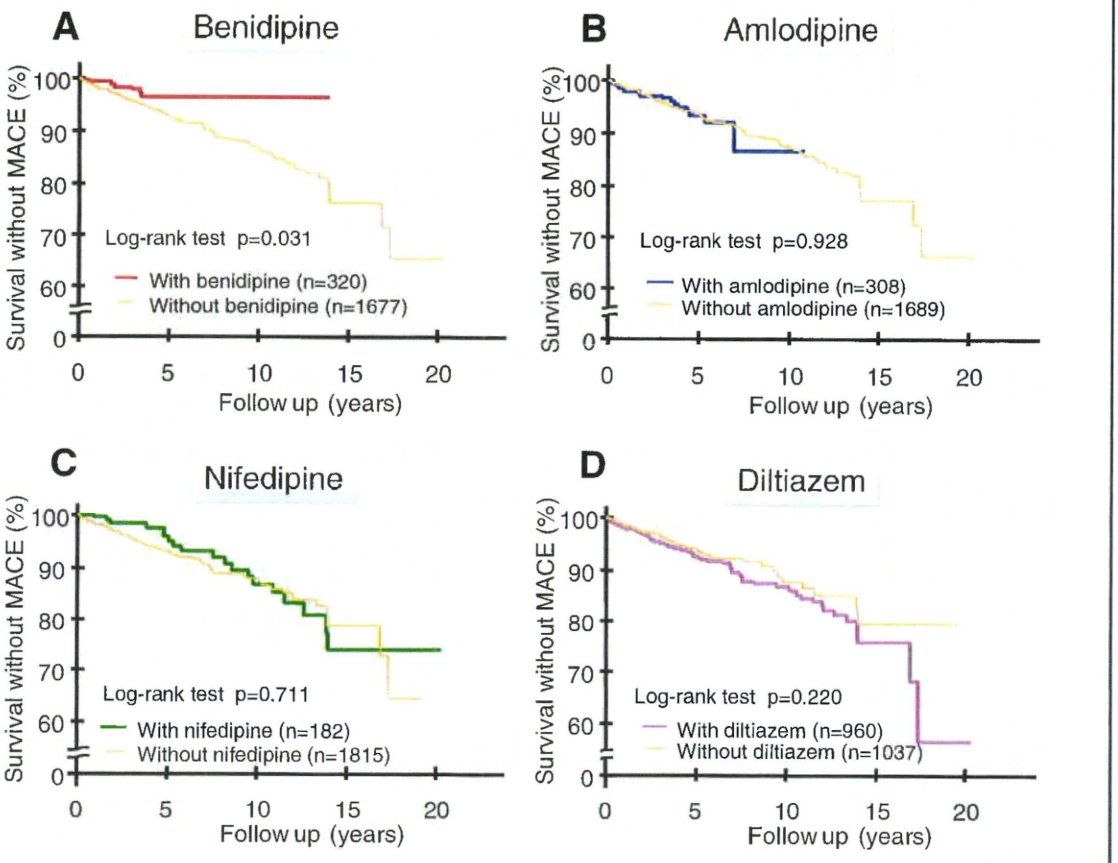

Figure 2. Kaplan–Meier curves for major adverse cardiovascular events (MACE)-free survival in vasospastic angina patients treated with each calcium channel blocker, (**A**) benidipine, (**B**) amlodipine, (**C**) nifedipine, and (**D**) diltiazem (n=1,997 in total).

Outcomes

The major outcome in this study was the occurrence of MACE, including cardiac death, MI (fatal and non-fatal), heart failure (death due to heart failure and heart failure requiring hospitalization), stroke (fatal and non-fatal), and aneurysm. The incidence of each MACE and the total number of deaths due to the event and its percentage were calculated for each CCB. We compared each MACE occurrence between the patients treated with each CCB and those without it. Because some differences were observed in the patient characteristics at baseline among the CCB groups, a search for factors (characteristics of patients and drugs used) that affected the occurrence of MACE was performed. The effect of the treatment with each CCB on the occurrence of MACE was then corrected for the factors that could have affected the occurrence of MACE.

Statistical Analysis

The major outcome of the present study was the occurrence of MACE. For each CCB, the period from the baseline day to the occurrence of MACE or the day of the last follow-up were plotted as a Kaplan–Maier curve, comparing the patients treated with each CCB and those without it. The curves were analyzed using the log-rank test. To search for factors related to the occurrence of MACE, the hazard ratio (HR) and its 95% confidence interval (CI) were calculated using Cox's regression analysis. The effect of the treatment with each CCB on the occurrence of MACE was corrected by Cox regression analysis using the factors that could affect the MACE occurrence (eg, smoking and a history of MI). In addition, in the 1,349 patients treated with a single CCB, the period until the occurrence of MACE was plotted in each CCB group as a Kaplan–Maier curve and compared using the log-rank test. All results are presented as means±SD. All statistical analyses were performed using SPSS for Windows ver. 11.0.1 (SPSS Japan Inc, Tokyo, Japan). Significance levels of 5% bilaterally and P-value less than 5% was considered to be statistically significant.

Results

Patient Characteristics

In the 4 papers used in the present meta-analysis, the diagnosis of VSA was made based on the 2008 Guidelines of the Japanese Circulation Society (**Table 1**).[15] As a result, the number of VSA patients reported by Io et al.[11] was changed from 1,047 to 879, and a total of 1,997 VSA patients with positive response to coronary spasm provocation tests were

finally analyzed. In addition, because the events used for the outcome evaluation varied slightly among the 4 papers, all of the events evaluated were handled as complex events. The follow-up period was recalculated according to the definition of MACE used in the present study, with a median follow-up period of 4.4 years (min–max: 0.2–21.9).

Table 2 shows the clinical characteristics of the VSA patients treated with each CCB. The age was higher in the benidipine group compared with other CCB groups. The prevalence of female patients was higher in the benidipine group compared with the nifedipine group. Among the major risk factors, hypertension was less frequent in the diltiazem group and hyperlipidemia was less frequent in the diltiazem or nifedipine groups compared with other CCB groups. Previous MI was observed more frequently in the nifedipine group. Regarding the concomitant drugs, nitroglycerin preparations were used less frequently in the benidipine group compared with other CCB groups, and nicorandil was more frequently used in the benidipine and nifedipine groups compared with other 2 groups. ARB were used more frequently in the amlodipine group compared with the nifedipine or diltiazem groups, while ACE inhibitors were less frequently used in the diltiazem group. Nifedipine was the long-acting form (twice a day or once a day), and diltiazem was slow-release preparation.

We also compared the reduction in the frequency of anginal attacks due to coronary spasm in the 4 CCB groups. We found that the frequencies of anginal attacks at follow up in the benidipine (0.7±1.1 attacks/month) and nifedipine (0.7± 1.6 attacks/month) groups were significantly lower than those in the amlodipine (1.4±1.6 attacks/month) and diltiazem (1.6± 5.9 attacks/month) groups (P<0.001, between each group, Mann–Whitney test).

Comparison of the Prognostic Effects of CCB

In the present study, MACE occurred in 143 patients, including cardiac death (n=36), AMI (n=51), heart failure (n=26), stroke (n=65) and aortic aneurysm (n=11). The total number of deaths was 118. Of these events, the number of each event observed in VSA patients treated with either of 4 CCB, ie, benidipine, amlodipine, nifedipine and diltiazem, was shown in Table 3. MACE occurred significantly less in the patients treated with benidipine compared with those without it (P= 0.031, log-rank test) (Figure 2). By contrast, no significant difference was noted between the patients treated with amlodipine, nifedipine or diltiazem and those without them (Figure 2). A search for the background factors that could have affected the occurrence of MACE showed significant differences with respect to age (10-year intervals), the presence of diabetes mellitus, the presence of CAD, and use of β-blockers (Table 4). The HR for the occurrence of MACE was significantly lower in the patients treated with benidipine compared to those without benidipine (HR=0.46, CI= 0.22–0.95, P=0.035) (Figure 3). The HR was also significantly lower in the patients treated with benidipine, even after correction for the background factors (HR=0.41, CI=0.20–0.85, P=0.016) (Figure 3). Again, no significant difference was noted with other 3 CCB (Figure 3).

Multivariate analysis showed that diabetes mellitus and CAD were independent risk factors for MACE. However, age and use of β-blockers had no significant impact on the

Table 4. Factors Influencing MACE in Patients With Vasospastic Angina (Univariate Cox Regression Analysis, n=1,997)

	HR (95%CI)	P value
Diabetes mellitus	2.02 (1.39–2.95)	<0.001
Coronary artery disease	1.97 (1.37–2.85)	<0.001
ARB	1.88 (0.91–3.89)	NS
β-blockers	1.69 (1.02–2.81)	0.043
Previous MI	1.48 (0.95–2.31)	NS
ARB/ACE inhibitors	1.48 (0.88–2.48)	NS
Nitrates	1.28 (0.90–1.82)	NS
Hypertension	1.23 (0.89–1.71)	NS
Age (every 10 years)	1.21 (1.04–1.42)	0.017
ACE inhibitors	1.17 (0.59–2.31)	NS
Nicorandil	1.13 (0.74–1.72)	NS
Smoking	1.04 (0.75–1.46)	NS
Anginal attacks (follow-up)	1.00 (0.99–1.01)	NS
Family history of IHD	0.92 (0.58–1.46)	NS
BMI, ≥22.5 kg/m²	0.85 (0.61–1.21)	NS
Hyperlipidemia	0.82 (0.57–1.18)	NS
Sex, female (vs male)	0.72 (0.48–1.07)	NS
Statins	0.65 (0.30–1.41)	NS
LVEF, <45%	0.55 (0.22–1.35)	NS

HR, hazard ratio; CI, confidence interval. Other abbreviatuions see in Tables 2,3.

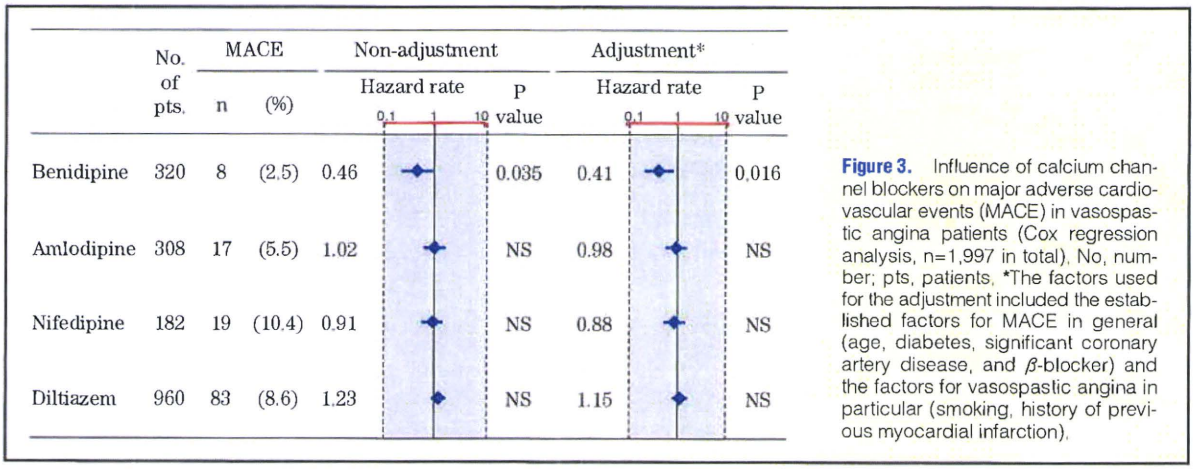

Figure 3. Influence of calcium channel blockers on major adverse cardiovascular events (MACE) in vasospastic angina patients (Cox regression analysis, n=1,997 in total). No, number; pts, patients. *The factors used for the adjustment included the established factors for MACE in general (age, diabetes, significant coronary artery disease, and β-blocker) and the factors for vasospastic angina in particular (smoking, history of previous myocardial infarction).

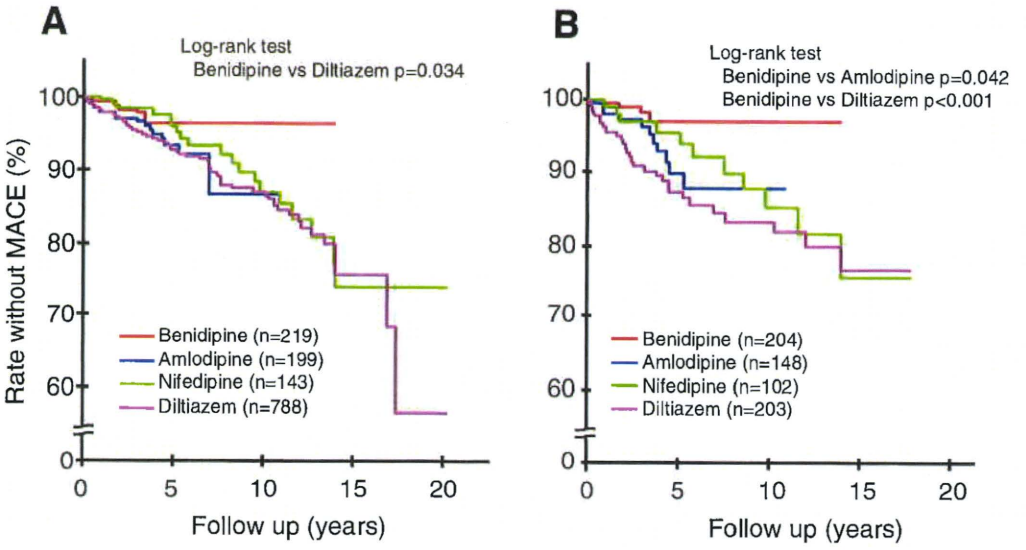

Figure 4. Kaplan–Meier curves for major adverse cardiovascular events (MACE)-free survival in vasospastic angina patients treated with each calcium channel blocker (**A**) before and (**B**) after the correction by the propensity scores.

occurrence of MACE. When the occurrence of MACE was compared among the 1,349 VSA patients treated with a single CCB, we again found that MACE occurred significantly less in the benidipine group (n=219) as compared with the diltiazem group (n=788) (P=0.034, log-rank test) (**Figure 4A**). We calculated the propensity score,[11] using the selection criteria for each agent to correct the bias of patients background. The factors of selection criteria included age, sex, hypertension, hyperlipidemia, and previous MI, and matching was performed. After correction by the propensity score, there was no significant difference in any factors among the 4 CCB groups, and again the incidence of MACE in the benidipine group (n=204) was significantly lower as compared with the diltiazem (n=203, P<0.001) and the amlodipine groups (n=148, P=0.042) (**Figure 4B**).

Discussion

The present meta-analysis with a total of 1,997 Japanese patients with VSA revealed positive coronary spasm provocation tests demonstrates that among the major CCB used for the treatment of VSA, benidipine might exert more beneficial prognostic effects as compared with other CCB. To the best of our knowledge, this is the first meta-analysis study that addresses the comparative prognostic effects of CCB in VSA patients.

Clinical Significance of Meta-Analysis for the Prognosis of VSA Patients

There have been several reports regarding the prognostic effects of CCB in patients with VSA.[8–14] However, all of these studies were retrospective in nature and therefore there could be some bias introduced when doctors chose the drugs. Nevertheless, these retrospective studies are valuable because they provide important data with VSA patients treated with different kinds of drugs. Moreover, although randomized controlled studies are considered superior to retrospective studies, one cannot exclude the bias introduced when doctors choose the patients to be enrolled. Therefore, we endeavored to evaluate the effects of CCB on the occurrence of MACE in a meta-analysis by using raw data and re-defining the study endpoint.

Prognostic Factors for the Incidence of MACE in VSA Patients

The present meta-analysis showed that diabetes mellitus, the presence of CAD, use of β-blockers and advanced age (every 10 years) were significant risk factors for MACE in VSA patients (**Table 4**). It was previously reported that the age older than 65 years was a risk factor for cardiovascular events.[10] However, in the present study, we found that advanced age, even less than 65 years, was a risk factor for MACE in VSA patients.

Diabetes mellitus is an established risk factor for cardiovascular events in VSA patients,[10,11] and insulin resistance associated with compensatory hyperinsulinemia is an independent risk factor for VSA.[16] Consistent with these findings, we found that diabetes mellitus is a risk factor for MACE. Indeed, the number of diabetic patients has been dramatically increasing in Japan,[17] indicating that special attention should be paid to diabetes mellitus in order to improve the prognosis of VSA patients.

It was repeatedly demonstrated that CAD is a prognostic factor in patients with rest angina and VSA.[1,10,11,13,18] Among these studies, Yamagishi et al emphasized that CAD is the most important prognostic factor affecting the prognosis of VSA patients, irrespective of the treatment with CCB.[18] The findings of the present study confirm that the risk of MACE is significantly increased by the presence of CAD, even in patients treated with CCB. Our findings also suggest the use of β-blockers might worsen the prognosis of VSA patients, which is consistent with an earlier report suggesting that β-blockers can worsen coronary vasospasm,[15] perhaps by

augmenting the effects of α-receptor stimulation.[19]

In the present study, multivariate analysis showed that diabetes mellitus and CAD were independent risk factors for MACE, although age and use of β-blockers had no significant impact on the occurrence of MACE. We also confirmed that the risk of cardiovascular events, such as sudden death, fatal and non-fatal MI, increased in VSA patients with significant coronary stenosis. Thus, careful observation is necessary for diagnosis and treatment of diabetes mellitus in VSA patients.

Prognostic Effects of CCB in VSA Patients

CCB are usually prescribed to VSA patients in an effort to prevent attacks of coronary vasospasm and related sudden cardiovascular death and other cardiac events.[7,9–14,18] However, only a few studies have compared the prognostic effects of CCB.[10–14] In these reports, the number of patients treated with CCB might not be high enough to draw a clear conclusion as to their prognostic effect in VSA patients. In the present study, we compared the prognostic effects of the 4 major CCB in a larger number of patients through a meta-analysis of the raw data. The result showed that benidipine had a significantly better prognostic effect as compared with amlodipine, nifedipine or diltiazem, while no differences were noted among the other 3 CCB.

Benidipine has been reported to be more selective for coronary artery smooth muscle cells compared with the other 3 CCB.[20,21] This higher selectivity of benidipine for coronary arteries might be involved not only in its inhibitory effects on coronary artery spasm, but also in its better prognostic effects. Moreover, the high affinity of benidipine for the coronary artery[22] might relate to its long-lasting effects independent of its blood concentration.[23] The beneficial prognostic effect of benidipine as compared with other CCB was first noted at sixth year, suggesting that vasculoprotective effects of but not anti-vasospastic effects of benidipine are involved (**Figure 2**). Indeed, it was recently shown that benidipine, but not diltiazem, improves vascular endothelial function assessed by flow-mediated dilation (FMD) in VSA patients[24] and that benidipine improves vascular functions, including FMD,[25] pulse wave velocity[26] and augmentation index[27] in patients with hypertension. We also previously reported that benidipine reduced MI size by increasing nitric oxide (NO) production and inhibiting free radical production in a rabbit model of MI.[28] It was reported that changing from diltiazem to benidipine reduced the frequency of anginal attacks associated with increased plasma NO levels in VSA patients.[29]

Study Limitations

The apparent limitation of the present study is that it is a meta-analysis of the retrospective cohort studies with VSA patients, where all of the patients were prescribed a CCB, at least at the time of follow-up investigation. However, we were unable to confirm treatment compliance (eg, the rate of drug cessation during the follow-up period) due to the unavailability of such data from the 4 studies.

Although the patient characteristics were corrected by propensity score matching in the present study, a prospective randomized controlled clinical trial should be conducted to compare the prognostic effects of CCB in patients with VSA. Furthermore, the information on the type of long-acting nifedipine (eg, twice a day type or once a day type) was unavailable in the present study. This point also remains to be examined in a future study.

Conclusions

The present meta-analysis with 1,997 VSA patients demonstrates that benidipine exerts more beneficial prognostic effects in Japanese patients with VSA as compared with the other 3 CCB, although this notion remain to be confirmed in future prospective randomized studies.

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Optical Coherence Tomography as a Novel Diagnostic Tool for Distal Type Chronic Thromboembolic Pulmonary Hypertension

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The new classification of pulmonary hypertension (PH) has been recently updated,[1] in which 5 major categories of the disorder have been classified. Among them, pulmonary arterial hypertension (PAH, class 1) and chronic thromboembolic PH (CTEPH, class 4) are characterized as having similar hemodynamic pulmonary circulation despite their different pathological vascular structures.

Optical coherence tomography (OCT) is an interferometer-based optical imaging modality that produces a 2-dimensional image of optical scattering from internal tissue microstructures with a high resolution of approximately 10–20 μm, 10-fold higher than that of intravascular ultrasound.[2–4] Here, we report the potential usefulness of OCT as a novel diagnostic tool for the differential diagnosis of distal type CTEPH from PAH.

The Ethics Committees of Tohoku University Hospital approved the study protocol and all patients provided written informed consent. We prospectively enrolled 31 consecutive patients with PH, including PAH (n=17), CTEPH (n=9), and normal hemodynamic subjects (control, n=5), who were

Table. Subject Characteristics

	Control	PAH	CTEPH	P value
n	6	18	9	
Age (years)	64.2±4.7	45.6±3.7	60.2±4.4	<0.05
Gender, n (%)				
Male	1 (17)	6 (33)	0 (0)	NS
Female	5 (83)	12 (67)	9 (100)	NS
Type of PH, n (%)				
IPAH	–	6 (33)	–	
CTD-PAH	–	9 (50)	–	
CHD-PAH	–	2 (11)	–	
Portal hypertension-PAH	–	1 (6)		
Hemodynamic variables				
PCWP (mmHg)	9±1	8±1	9±1	NS
Mean PAP (mmHg)	15±1	42±4	42±4	NS
CI ($L \cdot ml^{-1} \cdot m^{-2}$)	2.8±0.3	2.9±0.2	3.0±0.2	NS
Mean PVR ($dyne \cdot s^{-1} \cdot cm^{-5}$)	130±28	718±149	585±80	NS
OCT findings, n (%)				
Thrombus formation	0 (0)	0 (0)	4 (44)	<0.05
Luminal flaps	0 (0)	0 (0)	6 (67)	<0.01

Data are expressed as mean±SEM. Statistical analysis was performed between PAH and CTEPH, using unpaired t-test for continuous variables and chi-square test for categorical variables, using Stat View (SAS Institute, Cary, NC, USA).
PAH, pulmonary arterial hypertension; CTEPH, chronic thromboembolic pulmonary hypertension; PH, pulmonary hypertension; IPAH, idiopathic PAH; CTD, connective tissue disease; CHD, congenital heart disease; PCWP, pulmonary capillary wedge pressure; PAP, pulmonary arterial pressure; CI, cardiac index; PVR, pulmonary vascular resistance; OCT, optical coherence tomography.

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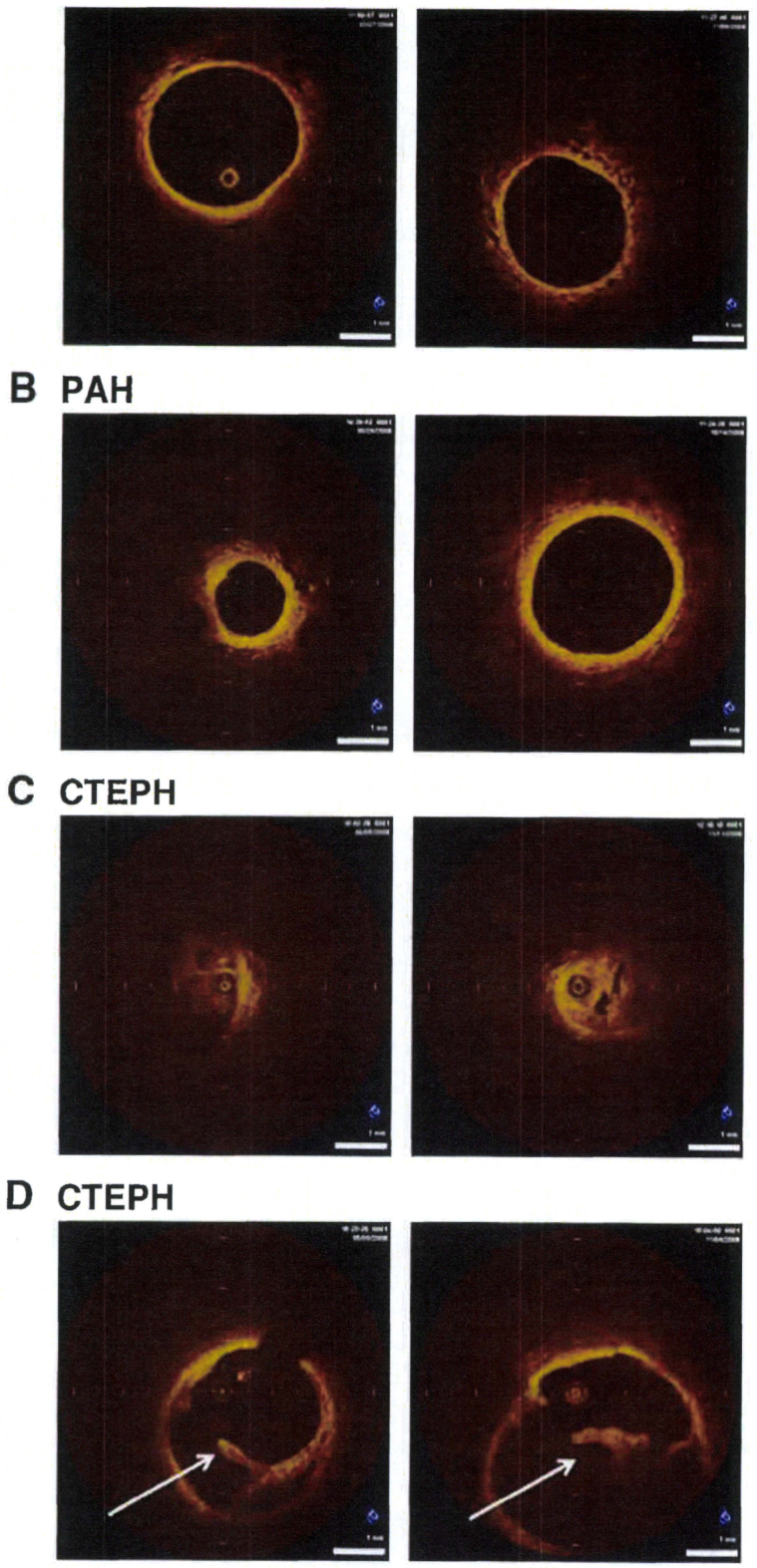

Figure. Optical coherence tomography (OCT) of the pulmonary arteries from (**A**) control subjects, (**B**) pulmonary artery hypertension (PAH) patients, and (**C**,**D**) chronic thromboembolic pulmonary hypertension (CTEPH) patients. The media of the pulmonary artery is thickened in (**B**) PAH, as compared with (**A**) controls. In contrast, in CTEPH, the pulmonary arteries have a very different OCT appearance with either (**C**) thrombotic occlusion and/or (**D**) luminal flaps (arrows). Bar, 1.0 mm.

admitted to Tohoku University Hospital from February 2009 to December 2009 and underwent right heart catheterization including OCT (**Table**). Pulmonary arteries >1 mm in diameter had no obstruction in the control or PAH subjects, although the media of the arteries appeared to be thickened in PAH subjects compared with controls (**Figures A,B**). In contrast, in all cases of CTEPH, we obtained completely different findings as compared with the control or PAH subjects; half of the CTEPH patients had occlusion of the pulmonary arteries, probably by thrombus (**Figure C**), and more than half of them had flaps in the lumen of the pulmonary arteries (**Figure D**; **Table**).

Pathohistological studies have demonstrated that idiopathic PAH is associated with abnormal vascular structures, including medial and/or intimal hypertrophy, concentric and/or eccentric intimal fibrosis, obstruction in the arterial lumen, and aneurysmal dilatation in vessels <300 mm in diameter.[5-7] In contrast, CTEPH is caused by the obstruction of pulmonary arteries by thrombus and is mainly observed in large vessels.[8,9] The present results indicate that OCT is a potentially useful tool for the differential diagnosis of distal type CTEPH from PAH.

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Hypertension Promotes Phosphorylation of Focal Adhesion Kinase and Proline-Rich Tyrosine Kinase 2 in Rats: Implication for the Pathogenesis of Hypertensive Vascular Disease

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Atherosclerosis is initiated by adhesion and infiltration of inflammatory leukocytes into the intima, where non-receptor protein tyrosine kinases, such as focal adhesion kinase (FAK) and proline-rich tyrosine kinase 2 (PYK2), play important roles as intracellular messengers of mechanical and biochemical signals. In the present study, we examined whether FAK and PYK2 are up-regulated by elevated blood pressure or circulating humoral factors in hypertension. We used a rat model of abdominal aortic banding that allows separate evaluation of elevated blood pressure (upper body) and circulating humoral factors (lower body). We obtained the proximal and distal aortas of the banding site, 6 hours, 3 days, and 1 and 4 weeks after the banding procedure, for evaluation of phosphorylation of FAK and PYK2 by Western blotting. Arterial pressure was significantly elevated only in the upper body throughout the experimental period. The expression of FAK and the FAK phosphorylation were significantly increased at 1 and 4 weeks only in the proximal aorta. This was also the case for the expression of total PYK2 and the PYK2 phosphorylation. In contrast, there was no significant change in FAK or PYK2 phosphorylation in the distal aorta, whereas plasma levels of angiotensin II were systemically elevated. In sham-operated rats, no change in FAK or PYK2 phoshorylation was noted in the proximal and distal aortas. These results indicate that phosphorylation of FAK and PYK2 is upregulated by elevated blood pressure but not by humoral factors in the rat aorta, demonstrating novel aspects of atherogenesis in hypertension.

Keywords: focal adhesion kinase; proline-rich tyrosine kinase 2; integrins, humoral factors; angiotensin II
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Atherosclerosis is the major cause of death in developed countries, which is initiated by adhesion and infiltration of inflammatory leukocytes into the intima (Ross 1999). In the process of atherosclerosis, inflammatory cytokines and adhesion molecules play important roles to develop atheroma that consists of fibrous cap and lipid core (Fukumoto et al. 1997; Ross 1999; Shimokawa 2000; Libby and Aikawa 2002; Fukumoto et al. 2004). It has been demonstrated that chronic hypertension accelerates vascular smooth muscle cells (VSMC) hypertrophy and extracellular matrix accumulation, which are substantially involved in the development of vascular remodeling and atherosclerosis (Lee et al. 1983; Schwartz et al. 1986; Bezie et al. 1998). Vascular remodeling induced by hypertension is mainly caused by two factors; increased mechanical stress and up-regulated humoral factors such as angiotensin II (Tamura et al. 1998; Shimokawa 2002). However, it remains to be examined to which extent these two mechanisms are involved in vascular remodeling in hypertension.

Recent studies have suggested that non-receptor protein tyrosine kinases play an important role in transducing mechanical stimuli to biochemical pathways by coupling with integrin (Burridge et al. 1992; Fukumoto et al. 1996; Sieg et al. 1998; Schlaepfer et al. 1999; Ostergaard and Lysechko 2005; Ramjaun and Hodivala-Dilke 2009). Among them, focal adhesion kinase (FAK) is a non-receptor protein tyrosine kinase that localizes to the point of cell contact with extracellular matrix and has been demonstrated to activate mitogen-activated protein kinases (MAPK) in response to integrin clustering in various types of cells (Burridge et al. 1992; Sieg et al. 1998; Schlaepfer et al. 1999; Ostergaard and Lysechko 2005; Vadali et al. 2007; Cai et al. 2008). Proline-rich tyrosine kinase 2 (PYK2) is another non-receptor protein tyrosine kinase that has a high sequence homology to FAK, sharing 45% amino-acid sequence identity (Sieg et al. 1998; Avraham et al. 2000;

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with a protein assay kit (Bio-Rad Laboratories, CA, USA). Twenty μg of each sample supplemented with 3X SDS were electrophoresed on a 7.5% SDS-polyacrylamide gel under a nonreducing condition. The protein on the gel was consequently transferred to the PVDF membrane (Bio-Rad Laboratories, CA, USA). The membrane was incubated over night with the primary antibodies used at a concentration of 1:1,000 (FAK, pY397, PYK2, pY407), 1:100 (integrin β1 and β3), followed by the second antibodies conjugating alkaline phosphatase. The membrane was developed with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium. Signals were visualized by the ECL detection system (Amersham Biosciences, Uppsala, Sweden) (Nakano et al. 2007; Do e et al. 2009).

Statistical Analysis

Statistical analyses were performed with StatView (StatView 5.0, SAS Institute Inc., Cary, NC). Comparisons of parameters between 2 groups under different conditions were made by 2-way ANOVA, followed by Scheffe's test. All values are presented as mean ± SEM. A value of $P < 0.05$ was considered to be statistically significant.

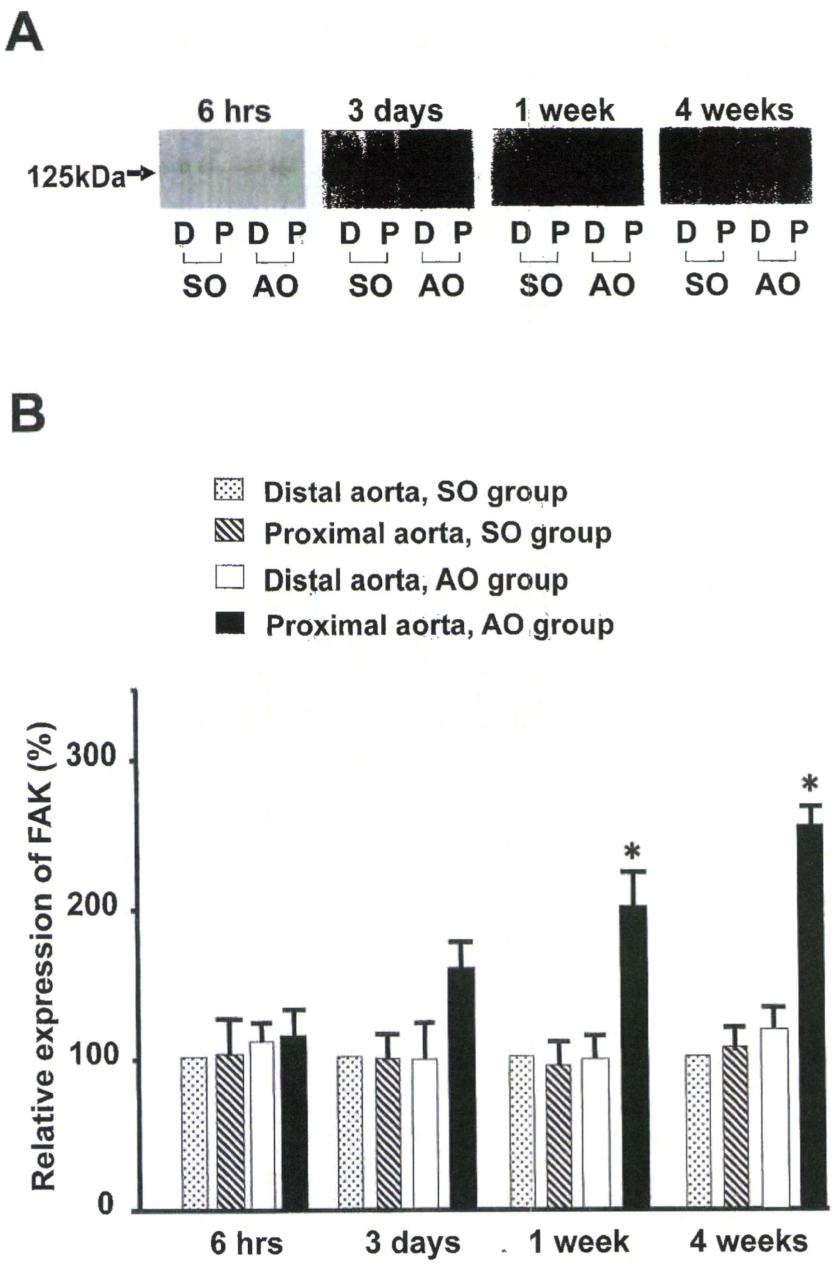

Fig. 1. FAK expression is enhanced by elevated blood pressure.
(**A**) Representative Western blot showing the expression of FAK in the proximal and distal aortas from the banding site in the aorta-banded (AO) and sham-operated (SO) groups at 6 hours, 3 days, 1 and 4 weeks after the operation. (**B**) Quantitative analysis of FAK expression. FAK expression was normalized to the level of expression in the distal aorta of the SO group at each period. Results are expressed as mean ± SEM. *$P < 0.05$ vs. other 3 groups.

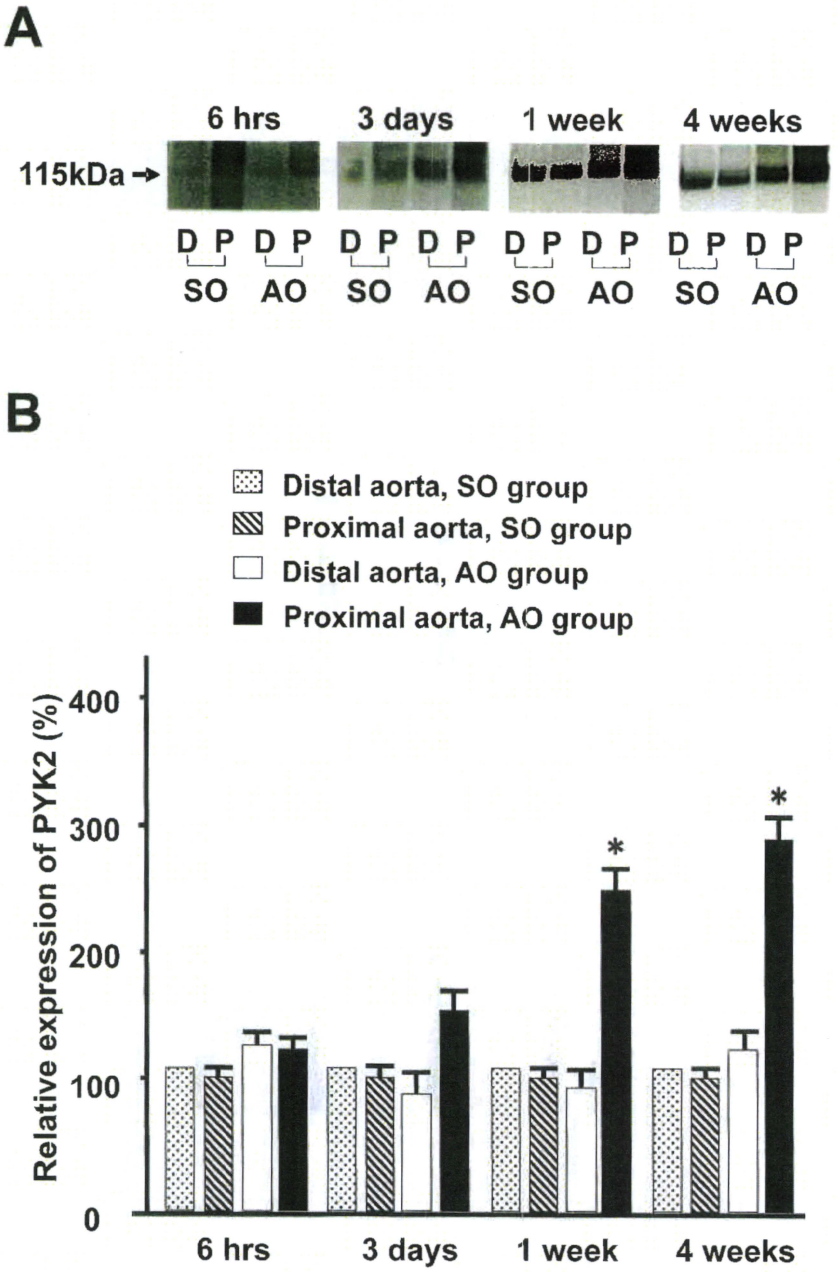

Fig. 3. PYK2 expression is enhanced by elevated blood pressure.
(**A**) Representative Western blot showing the expression of PYK2 in the proximal and distal aortas from the banding site in the aorta-banded (AO) and sham-operated (SO) groups at 6 hours, 3 days, and 1 and 4 weeks after the operation. (**B**) Quantitative analysis of PYK2. PYK2 expression was normalized to the level of expression in the distal aortas of the SO group at each period.
Results are expressed as mean ± SEM. $*P < 0.05$ vs. other 3 groups.

Expression and Phosphorylation of PYK2

Western blotting showed that the expression of PYK2 (Fig. 3) and PYK2 phosphorylation at tyrosine residue 402 (Fig. 4A-B) were significantly enhanced at 1 and 4 weeks only in the proximal aorta but not in the distal aorta in the AO group as compared with the SO group. In contrast, no changes were noted in the SO group. However, there was no significant difference in the ratio of PYK2 phosphorylation to PYK2 expression during the experiment (Fig. 4C).

Expression of Integrin β1 and β3

Western blotting showed that no changes were noted for integrin $\beta 1$ (Fig. 5) or integrin $\beta 2$ (Fig. 6) in both groups.

Discussion

The novel finding of the present study is that the expression and phosphorylation of FAK and PYK2 in the aortic wall are enhanced by mechanical stress but not by systemic humoral factors in rats in vivo and that altered

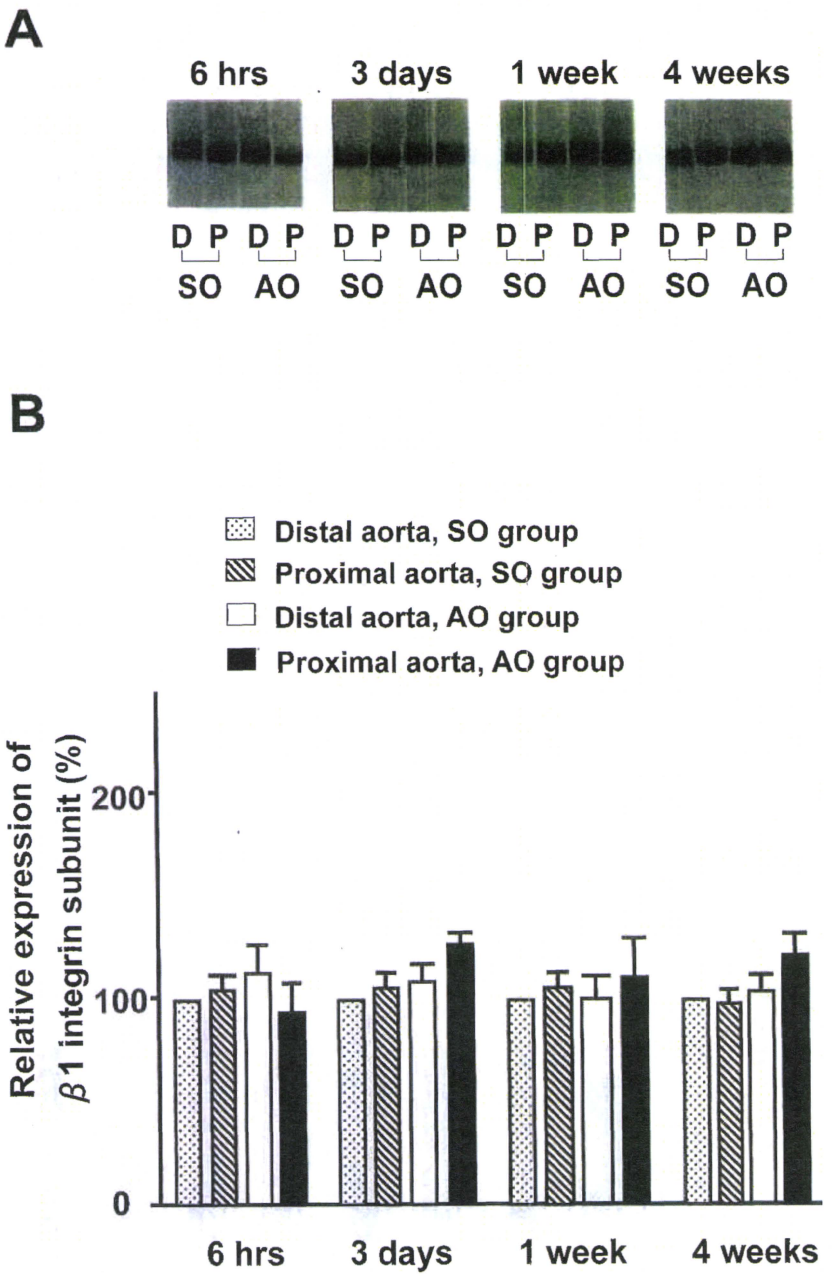

Fig. 5. Expression of $\beta 1$ integrin is unaltered in hypertension.
(**A**) Representative Western blot showing the expression of $\beta 1$-integrin subunit in the proximal and distal aortas from the banding site in the aorta-banded (AO) and sham-operated (SO) groups. (**B**) Quantitative analysis of $\beta 1$-integrin. Integrin expression was normalized to the level of expression in the distal aortas of the SO group at each period. Results are expressed as mean ± SEM. $^*P < 0.05$ vs. other 3 groups.

clustering of integrins (Schlaepfer et al. 1999). Mechanical stretch promotes FAK phosphorylation in various types of cells followed by activation of MAP kinase cascades (Li and Earp 1997; Smith et al. 1998; Torsoni et al. 2003). It has also been reported that FAK phosphorylation is enhanced by pressure stimulus in isolated mesenteric arteries (Rice et al. 2002). Taken together, these results indicate that FAK plays a central role in mechanical signal transduction by coupling with integrins.

Among several sites in the integrin-stimulated FAK tyrosine-phosphorylation, tyrosine residue 397 (Tyr397) is the most important phosphorylated site because as FAK with mutant Tyr397 failed to activate ERK stimulated by fibronectin (Schlaepfer and Hunter 1997). Furthermore, Try402 site of PYK2 is also known to be one of the major phosphorylation sites of PYK2, which plays an important role in the MAP kinase activation through the PYK2 signaling pathway (Avraham et al. 2000). In the present study, we were able to demonstrate that the expression of FAK and PYK2, as well as the phosphorylation of these molecules,

remain to be elucidated. In the present study, we thus used the rat model of abdominal aorta-banding with the plasma levels of circulating humoral factors but with different blood pressure between the upper and lower bodies and the results indicated that circulating humoral factors may not substantially be involved in the phosphorylation of FAK and PYK2 in the present model.

It was demonstrated that overexpression of FAK protein in cultured fibroblasts leads to activation of extracellular-related kinase (Schlaepfer et al. 1997) and that overexpression of PYK2 protein also accelerates phosphorylation of the downstream Src, Shc, and $p130^{CAS}$ (Li and Earp 1997; Sieg et al. 1998). These findings suggest that phosphorylation of tyrosine kinases may be enhanced not only by phosphorylation of the upstream tyrosine kinases but also by that of a total amount of protein expression of those molecules. In the present study, we were able to demonstrate that acute and chronic hypertension enhances the protein expression of FAK and PYK2 as well as the phosphorylation of these molecules in the proximal aorta of aorta-banded rats.

Limitations of the Study

Several limitations should be mentioned for the present study. First, we have demonstrated that the expression of β1- and β3-integrin was not increased in the proximal aorta, where the expression levels of FAK and PYK2 were increased. Although the property of interaction between integrin and FAK in cytoplasm has not been well documented (Giancotti and Ruoslahti 1999), it is recognized that integrins have three functional states, including latent, active and ligand-occupied states (Luque et al. 1996) and that overexpression of FAK leads to phosphorylation of MAP kinases through activating integrins (Schlaepfer and Hunter 1997). Thus, activation of integrins might be involved in phosphorylation of tyrosine-kinases in hypertension. This point remains to be examined in future studies. Second, although we performed the in vivo experiment to evaluate the total and phosphorylated FAK and PYK2, we did not evaluate the intracellular mechanisms by mechanical stretch or pressure overloaded. This point also remains to be examined in future studies.

Conclusions

In the present study, we are able to demonstrate that phosphorylation of FAK and PYK2 is enhanced by elevated blood pressure but not by humoral factors in the rat aorta, demonstrating novel aspects of atherogenesis in hypertension.

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