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ORIGINAL ARTICLE

Adding thiazide to a renin–angiotensin blocker improves left ventricular relaxation and improves heart failure in patients with hypertension

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Hypertension is associated with an increased risk of diastolic dysfunction. Angiotensin-converting enzyme inhibitors (ACEi) and angiotensin receptor blockers (ARB) have failed to show improvement in clinical outcomes for patients with diastolic dysfunction. In this study, we investigated the effect of changing an ACEi or ARB to a combination of losartan and hydrochlorothiazide (HCTZ) on left ventricular (LV) preload and relaxation in patients with hypertension and diastolic dysfunction. We enrolled 371 hypertensive patients with diastolic dysfunction who had not achieved their treatment goals with an ACEi or ARB. We switched the ACEi or ARB to losartan/HCTZ and followed the patients for 24 weeks. The primary end points were changes in septal mitral annular velocity during diastole (*e′*) and in the ratio of mitral inflow velocity to *e′* velocity (*E*/*e′* ratio) from baseline to the end of follow-up. Mean systolic and diastolic blood pressures (BP) decreased by 22 and 11 mm Hg, respectively, after changing from an ACEi or ARB to losartan/HCTZ. The *e′* velocity increased, and the *E*/*e′* ratio and brain natriuretic peptide level decreased significantly. High-sensitivity C-reactive protein also decreased significantly (0.50 *vs.* 0.29 mg dl^{-1}, $P<0.0001$). There were only slight or no changes in glucose levels, homeostasis model assessment insulin resistance (HOMA-R), uric acid and electrolytes after the drug change. Changing from an ACEi or ARB to losartan/HCTZ is associated with a reduction in BP, improvement in LV relaxation, improvement in heart failure state and attenuation of systemic inflammation with few adverse effects in patients with hypertension and diastolic dysfunction.
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INTRODUCTION

Diastolic dysfunction is the most common cause of heart failure (HF) in patients with hypertension.[1] Diastolic HF is associated with considerable morbidity and mortality, and the risk of adverse outcomes increases with the severity of diastolic dysfunction.[2] Patients with diastolic dysfunction have increased activation of the renin–angiotensin–aldosterone system, as do patients with systolic HF, which contributes to the pathogenesis and progression of the condition. However, angiotensin-converting enzyme inhibitors (ACEi) and angiotensin receptor blockers (ARB) have failed to show a benefit with regard to mortality or morbidity.[3–5] Thiazide is useful for reducing volume load in patients with diastolic dysfunction.[6] However, thiazide monotherapy has not been widely used in patients with diastolic dysfunction. Decreases in blood pressure (BP) are blunted by hypovolemia-induced activation of the renin–angiotensin–aldosterone system. Thiazide monotherapy involves risks of dysglycemia and/or dyslipidemia, and of elevation of C-reactive protein (CRP).[7] To resolve these problems, a small dose of thiazide in combination with an ACEi or ARB is given to allow for the full antihypertensive effect of thiazide and to minimize its adverse effects. However, it remains unknown whether this combination therapy can improve diastolic function or HF state and, if so, which factor has the principal role in the improvement of HF: reduction in left ventricular (LV) pre-load, improvement in its relaxation or both.

To answer these questions, we conducted a prospective, multicenter study to test the hypothesis that changing from an ACEi or ARB to losartan/hydrochlorothiazide (HCTZ) can improve LV diastolic function in patients with hypertension and diastolic dysfunction.

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The aims of this study were (1) to elucidate the impact of changing from an ACEi or ARB to losartan/HCTZ on LV preload, relaxation and severity of HF; (2) to determine the responders and non-responders to combination therapy; and (3) to determine the impact of this drug change on the metabolism of glucose, lipids, uric acid, electrolytes and CRP.

METHODS

Study population

Men and women from 20 to 80 years of age, with a history of stage 1 or 2 essential hypertension (mean BP measurement of >140 mm Hg systolic or >90 mm Hg diastolic), who were receiving treatment with an ACEi or ARB, were screened for inclusion by assessing their systolic and diastolic functions with echocardiography. Mitral annular relaxation velocity of $\leqslant 8\,cm\,s^{-1}$ was used to make a diagnosis of diastolic dysfunction. The exclusion criteria were an LV ejection fraction of <50%, septal mitral annular relaxation velocity $>8\,cm\,s^{-1}$, receiving treatment with diuretics and atrial fibrillation at baseline. The study protocol was approved by individual sites and written informed consent was obtained from all patients before any study procedures.

Study protocol

The patients were followed at least for 4 weeks to observe that target BP, <130/80 mm Hg, was not achieved with an ACEi or ARB. All patients underwent echocardiographic screening for systolic and diastolic functions before the start of treatment with losartan 50 mg/hydrochlorothiazide 12.5 mg. Then, the administration of ACEi or ARB was stopped, and ACEi or ARB was replaced with losartan/HCTZ. No other medications were changed throughout the study period. BP and heart rate were measured in a sitting position at each study visit. The adequacy of anti-hypertensive therapy was determined on the basis of measured BP. The use of concomitant antihypertensive medication was recorded at each study visit. If BP was not adequately controlled, add-on treatment other than diuretics was considered, but such subjects were dropped from this study. Patients were assessed at 4–8-week intervals for at least 24 weeks and underwent echocardiographic assessment at the end of the study. Blood and urine tests were also performed at baseline and at the end of the study.

Echocardiographic data analysis

From the mitral flow velocity pattern, we measured the peak velocities of E and A waves on mitral flow velocity, the ratio of their peak velocities (*E/A* ratio), the isovolumic relaxation time (IVRT) and the deceleration time of the E wave. Spectral pulsed-wave Doppler tissue interrogation of longitudinal mitral annular velocity was recorded throughout the cardiac cycle at the septal annulus in the apical four-chamber view. The peaks of apically directed systolic (*s′* velocity) and early diastolic (*e′* velocity) myocardial velocities were measured. Additional exploratory analyses included changes in chamber dimensions and the LV ejection fraction. LV mass was measured by using the American Society of Echocardiography-recommended formula and the end-systolic left atrial volume was measured by using the ellipsoid model.[8] Both values were indexed with body surface area in m^2.

The primary endpoints were changes in *e′* velocity and in the ratio of mitral inflow velocity to *e′* velocity (*E/e′* ratio) from baseline to follow-up. The secondary efficacy measures included differences in changes in BP, heart rate, wall thickness, the LV mass index and the left atrial volume index from baseline to follow-up. Blood samples were collected for measurements of brain natriuretic peptide (BNP) and high-sensitivity CRP (hsCRP), along with additional exploratory blood analyses, and urine was collected for measurement of albumin concentrations.

Statistical analysis

All of the results are expressed as the mean ± s.d. or proportions (%). Student's *t*-test was used for parametric data when a normal distribution and equal dispersion were recognized. The Welch's *t*-test was used when the variance was unequal. The paired *t*-test was used for comparing temporal changes in data. Univariate and multivariate analyses were performed to determine the independent factors related to changes in systolic BP, *e′* velocity and *E/e′* with changes from ACEi or ARB to losartan/HCTZ. Differences in the categorical data were analyzed by a χ^2 test and Fisher's exact test was used when appropriate. Differences were considered to be statistically significant when *P*-values were <0.05.

RESULTS

After echocardiographic screening, 415 patients with a history of treated or untreated hypertension were enrolled in this study between October 2008 and November 2009. Forty-four patients were excluded from this study, including 32 patients who were lost to follow-up, 7 patients who were excluded for protocol violation, 3 patients who were excluded for poor adherence and 2 patients who were excluded for atrial fibrillation at follow-up. Follow-up study was completed in 371 (89%) of the patients. Baseline characteristics, medication use and echocardiographic parameters before changing to losartan/HCTZ are shown in Table 1. The average interval between baseline and follow-up was approximately 6 months (range, 133–308 days). No patients died,

Table 1 Baseline demographic and clinical characteristics of study patients

Variables	*Population (n=371)*
Mean age (s.d.), years	67.5 (9.6)
Women, *n* (%)	135 (36)
Mean SBP (s.d.), mm Hg	155 (15)
Mean DBP (s.d.), mm Hg	87 (11)
Mean pulse (s.d.), b.p.m.	73 (11)
Weight (s.d.), kg	64.8 (11.4)
Body mass index (s.d.), $kg\,m^{-2}$	25.3 (3.4)
NYHA functional class	
Class I, *n* (%)	228 (61.5)
Class II, *n* (%)	138 (37.2)
Class III, *n* (%)	5 (1.3)
Diabetes, *n* (%)	106 (28.6)
Myocardial infarction, *n* (%)	19 (5.1%)
Mean eGFR (s.d.), $ml\,min^{-1}$ per $1.73\,m^2$	68.7(17.7)
Antihypertensive medication, n (%)	
ARB, *n* (%)	324 (87.3)
ACEi, *n* (%)	47 (12.7)
Other hypertensive medications, n (%)	
Calcium channel blocker, *n* (%)	171 (46.9)
Beta-blocker, *n* (%)	89 (24.0)
Alpha-blocker, *n* (%)	12 (3.2)
Echocardiographic parameters	
LVDd (s.d.), mm	47.4 (0.45)
LVDs (s.d.), mm	29.5 (0.45)
Septal wall thickness (s.d.), mm	10.0 (1.6)
LVMI (s.d.), $g\,m^{-2}$	102 (22)
LA dimension, mm	40.9 (8.1)
LA volume index, (s.d.), $ml\,m^{-2}$	25.4 (9.3)
LVEF (s.d.), %	67.2 (9.1)

Abbreviations: ACEi, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; bpm, beats per minute; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; LA, left atrial; LVDd, left ventricular end-diastolic dimension; LVDs, left ventricular end-systolic dimension; LVEF, left ventricular ejection fraction; LVMI, left ventricular mass index; SBP, systolic blood pressure; s.d., standard deviation.

developed HF or cardiovascular events or were admitted to the hospital in the course of the study.

The effect of change from ACEi or ARB to losartan/HCTZ on BP, echocardiographic parameters and laboratory data

At follow-up, the mean systolic and diastolic BPs and pulse pressure decreased from baseline by 23, 11 and 12 mm Hg, respectively (Table 2). Heart rate decreased by 2 beats min^{-1}. Additional hypertensive medications were given to only 26 patients (7%) and the medications given to most patients were calcium channel blockers (24 patients (6.5%)). The LV wall thickness, LV mass index and left atrial volume were significantly decreased 6 months after changing to losartan/HCTZ. There were no changes in peak E, deceleration time or *E/A* ratio. The *e'* velocity significantly increased (5.5–6.5 cm s^{-1}, $P<0.0001$), implying an improvement in LV relaxation. The *E/e'* ratio significantly decreased, from 12.1 to 10.6 at follow-up ($P<0.0001$), indicating a decrease in LV preload. IVRT also decreased significantly (120–113 ms, $P<0.0001$). Although there was no change in the LV ejection fraction after changing to losartan/HCTZ, *s'* velocity significantly increased, suggesting improvement in LV long-axis contractile function.

BNP levels decreased after changing from ACEi or ARB to losartan/HCTZ (48.6 *vs.* 36.6 pg dl^{-1}, $P=0.0006$). Interestingly, hsCRP levels showed a significant reduction after changing to losartan/HCTZ. Urine albumin concentrations also decreased at follow-up. Microalbuminemia ($\geqslant$30 mg per g Cr) was present in 73 patients (19.7%) at baseline, but was present in only 49 patients (13.2%) after 6 months of changing to losartan/HCTZ. There were slight increases in serum creatinine and blood urea nitrogen, and a slight decrease in the estimated glomerular filtration rate at follow-up. In all patients, uric acid did not show a significant change after changing to losartan/HCTZ. In patients with low uric acid levels (<7.0 mg dl^{-1}) at baseline

Table 2 Changes in hemodynamic and echocardiographic parameters with changes from ACEi or ARB to losartan/HCTZ

Variables	*ACEi or ARB*	*6 months of losartan/HCTZ*	P-*value*
SBP, mm Hg	155 (15)	132 (12)	<0.0001
DBP, mm Hg	87 (11)	76 (10)	<0.0001
Pulse pressure, mm Hg	68 (16)	56 (12)	<0.0001
Pulse, b.p.m.	73 (11)	71 (11)	0.0006
Echo parameters			
e' velocity, cm s^{-1}	5.4 (1.4)	6.5 (1.8)	<0.0001
s' velocity, cm s^{-1}	7.7 (2.6)	8.2 (2.6)	<0.0001
E/e' ratio	12.1 (3.8)	10.6 (3.7)	<0.0001
IVRT, ms	120.3 (31.1)	112.7 (27.2)	<0.0001
DT, ms	238.3 (54.6)	233.7 (47.8)	0.1214
E velocity, m s^{-1}	64.0 (15.8)	63.9 (14.7)	0.9118
A velocity, m s^{-1}	82.3 (17.7)	79.3 (17.0)	<0.0001
E/A ratio	0.80 (0.21)	0.83 (0.22)	0.0013
Septal thickness, mm	10.0 (1.6)	9.7 (1.5)	<0.0001
LVEF, %	67.2 (9.1)	67.0 (10.6)	0.6504
LVMI g m^{-2}	102 (22)	96 (22)	<0.0001
LA volume index, ml m^{-2}	25.4 (9.3)	23.6 (8.7)	<0.0001
Laboratory data			
BNP, pg dl^{-1}	48.6 (73.2)	36.6 (55.7)	0.0006
HsCRP, mg dl^{-1}	0.50 (0.62)	0.29 (0.73)	<0.0001
Urine albumin, mg per g Cr	71.8 (219.6)	47.0 (228.3)	0.0010
BUN, mg dl^{-1}	17.0 (4.5)	18.2 (5.3)	<0.0001
Serum creatinine, mg dl^{-1}	0.83 (0.24)	0.86 (0.25)	0.0001
eGFR, ml min^{-1}	68.7(17.7)	66.6 (18.3)	0.0001
Uric acid, mg dl^{-1} (all)	5.9 (1.4)	6.0 (1.5)	0.7318
Uric acid (<7.0), mg dl^{-1}	5.3 (1.0)	5.6 (1.4)	<0.0001
Uric acid ($\geqslant$7.0), mg dl^{-1}	7.7 (0.7)	6.9 (1.2)	<0.0001
Na, mEq dl^{-1}	141.7 (1.9)	141.3 (2.4)	0.0032
K, mEq dl^{-1}	4.3 (0.4)	4.1 (0.4)	<0.0001
Cl, mEq dl^{-1}	104.0 (2.4)	103.0 (3.1)	<0.0001
Fasting BS, mg dl^{-1}	111.1 (26.8)	110.9 (28.2)	0.8732
HbA1c, %	6.19 (0.74)	6.14 (0.75)	0.2368
HOMA-R	3.73 (5.01)	3.53 (4.61)	0.5218
Total cholesterol, mg dl^{-1}	202 (31)	195 (31)	<0.0001
HDL cholesterol, mg dl^{-1}	55 (14)	54 (14)	0.0011
Triglyceride, mg dl^{-1}	157 (108)	141 (66)	0.0004

Abbreviations: BNP, brain natriuretic peptide; BS, blood sugar; BUN, blood urea nitrogen; Cr, creatinine; DBP,diastolic blood pressure; DT, deceleration time; eGFR, estimated glomerular filtration rate; HDL, high density lipoprotein; hsCRP, high sensitivity C-reactive protein; IVRT, isovolumetric relaxation time; LA, left atrial; LVEF, left ventricular ejection fraction; LVMI, left ventricular mass index; SBP, systolic blood pressure
All values are expressed as the mean (s.d.).
HOMA-R: (IRI*FBS/405).

it increased slightly, and it decreased in patients with a high uric acid level ($\geqslant 7.0\,mg\,dl^{-1}$) at baseline. There were no changes in fasting glucose levels, hemoglobin A1c, fasting insulin levels or homeostasis model assessment insulin resistance (HOMA-R) after changing to losartan/HCTZ.

Factors related to changes in *e'* velocity and *E/e'* ratio

We studied the impact of the magnitude of reduction in systolic BP with drug changes on *e'* velocity and the *E/e'* ratio. A greater reduction in systolic BP was associated with a greater increase in *e'* velocity and a greater reduction in the *E/e'* ratio (Figure 1). There was a close relationship between changes in *s'* velocity and changes in *e'* velocity (Figure 2).

We analyzed baseline factors related to the magnitude of changes in systolic BP, *e'* velocity and the *E/e'* ratio with a change from ACEi or ARB to losartan/HCTZ (Table 3). The factors included age, sex, diabetes, body mass index, New York Hypertension Association (NYHA) class, systolic BP, pulse pressure, heart rate, hsCRP, HOMA-R, BNP, estimated glomerular filtration rate, LV mass index, left atrial volume index and microalbuminemia. Multivariate analysis showed that baseline systolic BP and NYHA class were independent factors ($P<0.05$) related to the magnitude of reduction in systolic BP after changing to losartan/HCTZ. Multivariate analysis also showed that age, body mass index, NYHA class, systolic BP and hsCRP were independent factors related to changes in *e'* velocity after changing to losartan/HCTZ. NYHA class, systolic BP and hsCRP were independent factors related to the magnitude of changes in the *E/e'* ratio after changing to losartan/HCTZ.

As baseline hsCRP is an independent factor related to changes in *e'* velocity and the *E/e'* ratio, we compared temporal changes in these parameters in patients with high hsCRP ($\geqslant 2.0\,mg\,dl^{-1}$) and those with low hsCRP ($<0.2\,mg\,dl^{-1}$) (Figure 3). Reductions in the *E/e'* ratio and increases in *e'* velocity were greater in patients with high hsCRP at baseline. We studied the impact of NYHA class on changes in *e'* velocity and the *E/e'* ratio after changing to losartan/HCTZ. Our data showed that increases in *e'* velocity and decreases in the *E/e'* ratio were greater in patients with NYHA class 2 or 3 than in patients with NYHA class 1.

Adverse events. Adverse events were observed in 22 patients. The adverse events included photosensitivity or skin eruption (11 patients), dizziness (5 patients), general fatigue (4 patients), an increase in BP (1 patient) and worsening of renal function (1 patient). Losartan/HCTZ was withdrawn in 14 patients and they were dropped from the study.

DISCUSSION

We showed that changes from ACEi or ARB to losartan/HCTZ further lowered BP, improved LV relaxation (an increase in *e'* velocity), reduced LV preload (a decrease in the *E/e'* ratio), and improved HF state (a decrease in BNP) in patients with hypertension and diastolic dysfunction. The drug change was also associated with reductions in hsCRP. We first documented that the change from ACEi or ARB to losartan/HCTZ could improve LV relaxation and HF in patients with diastolic dysfunction, and that these improvements were associated with attenuation of systemic inflammation.

The optimization of hemodynamics is achieved primarily by reducing cardiac preload and afterload in patients with diastolic dysfunction. An ACEi or ARB may affect myocardial relaxation and compliance by reducing BP and arterial stiffness and by reducing interstitial collagen deposition, but it cannot reduce LV preload. Diuretics are effective for reducing LV preload. Our results showed

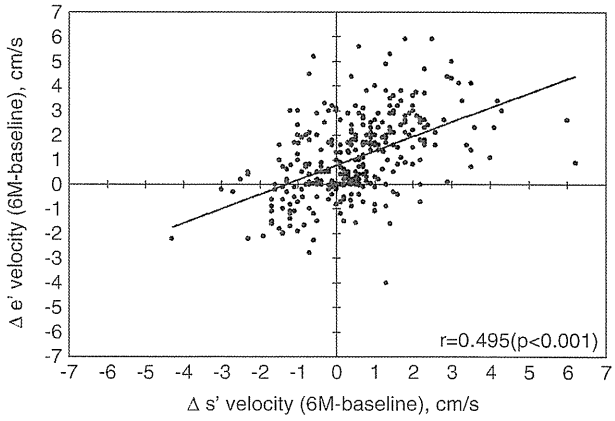

Figure 2 Relationship between changes in *e'* velocity and changes in *s'* velocity. There was a close relationship between changes in *s'* velocity and changes in *e'* velocity.

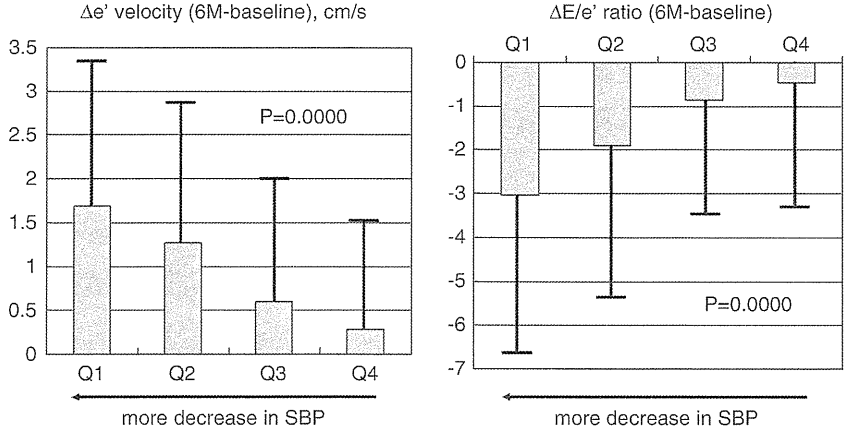

Figure 1 Changes in *e'* velocity and *E/e'* ratio for each quartile of systolic BP decrease after change to losartan/HCTZ. A greater reduction in systolic BP was associated with a greater increase in *e'* velocity and a greater reduction in *E/e'*. The range of decrease in systolic BP was 72–35 mm Hg in Q1, 34–22 mm Hg in Q2, 21–13 mm Hg in Q3 and 12–27 mm Hg in Q4. Data are expressed as mean ± s.d.

Table 3 Predictors of changes in SBP, *e'* velocity and *E/e'* ratio after changing from ACEi or ARB to losartan/HCTZ

	Changes in SBP		*Changes in e' velocity*		*Changes in E/e' ratio*	
	Univariate analysis P-*value*	*Multivariate analysis* P-*value*	*Univariate analysis* P-*value*	*Multivariate analysis* P-*value*	*Univariate analysis* P-*value*	*Multivariate analysis* P-*value*
Age	0.9693		0.0009	0.0001	0.0923	
BMI	0.0994		0.0281	0.0065	0.0341	0.2024
NYHA 1 *vs.* 2 or 3	0.0000	0.0030	0.0000	0.0000	0.0000	0.0010
SBP	0.0000	0.0000	0.0000	0.0043	0.0000	0.0189
HsCRP	0.0000	0.9939	0.0000	0.0001	0.0000	0.0000

Abbreviations: BMI, body mass index; HCTZ, hydrochlorothiazide; hsCRP, high sensitivity C-reactive protein; NYHA, New York hypertension association; SBP, systolic blood pressure

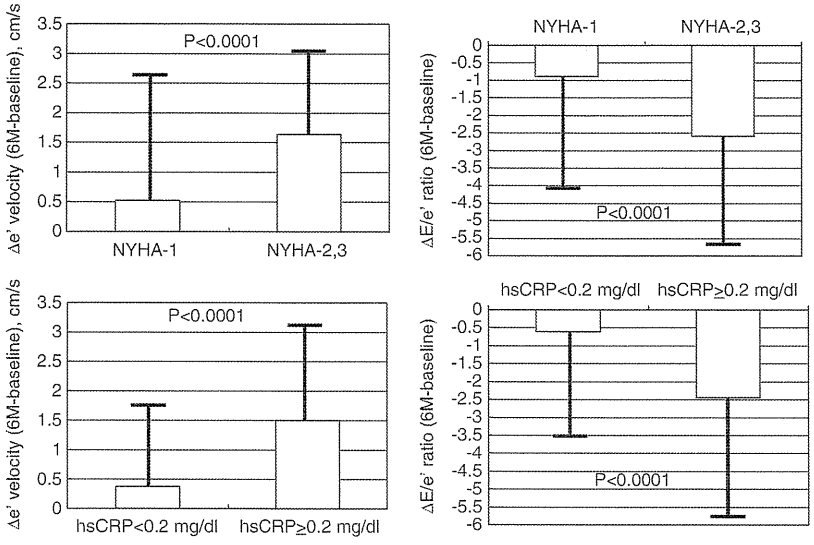

Figure 3 Comparisons of temporal changes in *e'* velocity and *E/e'* ratio between NYHA class, high and low hsCRP, and high and low HOMA-R. The magnitude of changes in *e'* velocity and the *E/e'* ratio was higher in patients with NYHA-2 or 3 than those with NYHA-1. The magnitude of changes in *e'* velocity and the *E/e'* ratio was greater in high hsCRP patients than in low hsCRP patients. The magnitude of changes in *e'* velocity was greater in patients with high HOMA-R than in those with low HOMA-R. However, there was no difference in the *E/e'* ratio between the two groups. A full color version of this figure is available at the *Hypertension Research* journal online.

that changing an ACEi or ARB to losartan/HCTZ is associated with a reduction of the *E/e'* ratio and an increase in *e'* velocity. A decrease in IVRT after the drug change also suggests an improvement in LV relaxation. IVRT is sensitive to LV preload and relaxation. In the case of reduced preload, IVRT decreases only when LV relaxation improves, as shown in this study. The combination of improvement in LV relaxation and a decrease in preload can be attributed to a reduced left atrial volume index and BNP level at follow-up.

There are several mechanisms by which *e'* velocity can increase with changes from ACEi or ARB to losartan/HCTZ. Reductions in systolic BP themselves can be associated with increases in *e'* velocity.[9–11] The CALVLOC (Clinical Impact of Azelnidipine on Left VentricuLar Diastolic Function and OutComes in Patients with Hypertension) trial documented that a reduction in systolic BP with a calcium channel blocker, azelnidipine, was associated with an increase in *e'* velocity.[10] This study showed that a greater increase in *s'* velocity after changing to losartan/HCTZ was associated with a greater increase in *e'* velocity. Many patients with diastolic dysfunction may have subtle abnormalities of systolic contractile function. The LV longitudinal contractile function is a primary contributor to LV pumping and is particularly sensitive to the effects of afterload.[12] It is speculated that further reduction in afterload with losartan/HCTZ can improve LV longitudinal contractile function (an increase in *s'* velocity), resulting in improvement of LV relaxation. Reduction of the LV mass index can also contribute to improvement in LV relaxation.[13]

In this study, hsCRP significantly decreased with changes from ACEi or ARB to losartan/HCTZ. CRP level is a marker of systemic inflammation. Higher levels of CRP are associated with higher BP.[14] CRP levels are also elevated in patients with diastolic dysfunction, and they correlate with disease severity, as well as LV preload.[15] The mechanism of CRP elevation in patients with diastolic dysfunction has not been elucidated. Organ congestion and hypoperfusion might influence the secretion of interleukin-6 by hepatic, renal, endothelial, mononuclear and even cardiac myocytes.[16] Elevated inflammatory markers have been associated with an increased risk of developing symptomatic HF.[17] CRP may be an active participant in the development of diastolic dysfunction. CRP can inhibit nitric oxide production, impede endothelial function and activate the complement cascade.[18] Interleukin-6, which affects CRP, has been shown to promote myocyte hypertrophy.[19] Reductions in BP and LV preload with changes to losartan/HCTZ may contribute to a reduction in CRP. Therefore, the attenuation of the systemic inflammatory response with changes to losartan/HCTZ may contribute to the improvement of LV diastolic function.

Previous studies have suggested that BNP,[20] the left atrial volume index,[21] the LV mass index[22] and hsCRP are useful for risk stratification in patients with HF. A lower BNP, left atrial volume index or LV mass index is associated with better clinical outcomes, and lower rates of morbidity and mortality. These parameters are now regarded as excellent surrogate end points for assessing the utility of therapeutic interventions. Our results showed that BNP, the left atrial volume index and the LV mass index significantly decreased with changes from ACEi or ARB to losartan/HCTZ. Therefore, the combination of an ARB and a small dose of thiazide might be a good therapeutic choice for reducing hospitalization due to HF, and mortality in patients with hypertension and diastolic dysfunction.

Much of the criticism against thiazides has been directed toward their adverse-effect profile. Thiazides can reduce the excretion of uric acid and thereby increase its plasma level. Our results, however, showed that there was no change in uric acid levels with changes from ACEi or ARB to losartan/HCTZ. This finding was because of the potential of losartan to augment the excretion of uric acid.[23] Some patients with diastolic dysfunction are sensitive to preload reduction and may develop severe pre-renal azotemia. In this study, blood urea nitrogen and creatinine levels showed only slight increases with changes to losartan/HCTZ. New-onset diabetes has been reported in patients receiving thiazides.[24] Maintaining potassium homeostasis is essential because epidemiologic evidence implicates hypokalemia in the pathogenesis of thiazide-induced dysglycemia.[25] In this study, there were no changes in potassium levels or levels of blood glucose, HbA1c, serum insulin and HOMA-R after changing to losartan/HCTZ.

Limitations

Some limitations should be noted. This study was a single-arm trial. The main objective of this study was not to monitor clinical outcomes, but to detect changes in objective parameters to assess the change made upon LV diastolic function after changing from ACEi or ARB to losartan/HCTZ. We did not simply add a small dose of HCTZ to each ACEi or ARB, but changed them to losartan/HCTZ to improve patient adherence. Only three patients dropped out because of poor adherence. Most of the patients were mildly to moderately hypertensive and without clinical HF. The findings in this study may not be applicable to patients with severe diastolic dysfunction. In this study, however, the magnitude of increase in e' velocity was greater and the magnitude of decrease in the E/e' ratio was also greater in patients with symptomatic HF. It is not clear whether an increase in e' velocity by 1.0 cm s^{-1} or decrease in the E/e' ratio by 1.5 is clinically meaningful or whether it would be associated over time with improvement in outcomes.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Pro-apoptotic effects of imatinib on PDGF-stimulated pulmonary artery smooth muscle cells from patients with idiopathic pulmonary arterial hypertension ☆

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ABSTRACT

Background: Remodeling of the pulmonary artery by an inappropriate increase of pulmonary artery smooth muscle cells (PASMCs) is problematic in the treatment of idiopathic pulmonary arterial hypertension (IPAH). Effective treatment that achieves reverse remodeling is required. The aim of this study was to assess the pro-apoptotic effects of imatinib, a platelet-derived growth factor (PDGF)-receptor tyrosine kinase inhibitor, on PASMCs obtained from patients with IPAH.

Methods: PASMCs were obtained from 8 patients with IPAH undergoing lung transplantation. Cellular proliferation was assessed by ^{3}H-thymidine incorporation. Pro-apoptotic effects of imatinib were examined using TUNEL and caspase-3,7 assays and using transmission electron microscopy.

Results: Treatment with imatinib (0.1 to 10 μg/mL) significantly inhibited PDGF-BB (10 ng/mL)-induced proliferation of PASMCs from IPAH patients. Imatinib (1 μg/mL) did not induce apoptosis in quiescent IPAH-PASMCs, but it had a pro-apoptotic effect on IPAH-PASMCs stimulated with PDGF-BB. Imatinib did not induce apoptosis in normal control PASMCs with or without PDGF-BB stimulation. PDGF-BB induced phosphorylation of Akt at 15 min, and Akt phosphorylation was inhibited by imatinib in IPAH-PASMCs. Akt-I-1/2 (1 μmol/L), an Akt inhibitor, in the presence of PDGF-BB significantly increased apoptotic cells compared with the control condition. Thus, Akt-I-1/2 could mimic the effects of imatinib on PASMCs.

Conclusion: Imatinib has anti-proliferative and pro-apoptotic effects on IPAH-PASMCs stimulated with PDGF. The inhibitory effect of imatinib on Akt phosphorylation induced by PDGF plays an important role in the pro-apoptotic effect.

1. Introduction

Idiopathic pulmonary arterial hypertension (IPAH) is a progressive disease characterized by progressive elevation of pulmonary vascular resistance and pulmonary artery pressure. Increased pulmonary vascular resistance is induced by pulmonary vasoconstriction, vascular remodeling by intimal and medial hypertrophy, and thrombosis [1,2]. Pulmonary vascular medial hypertrophy is caused by an inappropriate increase in pulmonary artery smooth muscle cells (PASMCs). Treatment with several vasodilators such as calcium channel blockers, prostaglandin I_2 and endothelin receptor antagonists was found to improve survival of patients with IPAH, but 5-year survival remains at 50% [3,4]. Effective treatment that achieves reverse remodeling is needed. This will require anti-proliferative and pro-apoptotic agents for PASMCs.

We have reported that platelet-derived growth factor (PDGF)-BB stimulation causes a higher growth rate of cultured PASMCs from patients with IPAH than that of control cells [5–7]. Recently, the use of a PDGF-receptor inhibitor such as imatinib (STI571) is starting to garner attention as a targeted therapy for pulmonary hypertension (PH) [8–11]. Imatinib is a drug used to treat certain types of cancer such as chronic myelogenous leukemia and gastrointestinal stromal tumors. In laboratory settings, imatinib is used as an experimental agent to suppress PDGF by inhibiting PDGF receptor ß (PDGF-Rß). It is an agent that acts by specifically inhibiting a certain enzyme, tyrosine kinase, that

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Table 1
Clinical data of patients with IPAH.

Patient	Time	Sex	Age	PAP (s/d/m) (mmHg)	mRAP (mmHg)	CI (L/min/m^2)	PVR (dyn/s/cm^5)	BNP (pg/dL)
1	Prior to drug therapy	F	7	150/72/98	4	3.8	1918	136
	Prior to transplantation		13	99/59/72	15	2.3	2779	334
2	Prior to drug therapy	F	28	88/40/59	10	1.9	1416	408
	Prior to transplantation		31	73/30/48	1	2.1	1199	325
3	Prior to drug therapy	F	10	118/67/84	14	2	NA	NA
	Prior to transplantation		13	111/49/67	10	1.7	2438	203
4	Prior to drug therapy	F	NA	NA	NA	NA	NA	NA
	Prior to transplantation		28	113/36/66	7	1.8	3340	50
5	Prior to drug therapy	M	16	163/71/106	2	1.7	2267	14
	Prior to transplantation		20	70/40/50	2	3.3	808	18
6	Prior to drug therapy	F	39	74/23/42	3	2.6	NA	NA
	Prior to transplantation		43	107/47/72	15	2.4	3056	622
7	Prior to drug therapy	F	13	96/50/68	4	2.3	1495	411
	Prior to transplantation		16	83/51/65	8	2.5	784	216
8	Prior to drug therapy	M	NA	NA	NA	NA	NA	NA
	Prior to transplantation		11	130/51/80	9	1.9	2629	420
Mean ± SE	Prior to drug therapy		19 ± 5	mPAP: 76 ± 10	6 ± 2	2.4 ± 0.3	1774 ± 198	242 ± 100
	Prior to transplantation		22 ± 4	mPAP: 65 ± 4	8 ± 2	2.3 ± 0.2	2129 ± 366	273 ± 70

M: male, F: female, PAP: pulmonary artery pressure, s/d/m: systolic/diastolic/mean, mRAP: mean right atrial pressure, CI: cardiac index, PVR: pulmonary vascular resistance, BNP: plasma concentration of brain natriuretic peptide, NA: not available.

is characteristic of a particular cancer cell, rather than non-specifically inhibiting the proliferation of and killing all rapidly dividing cells. Schermuly et al. reported that imatinib reverses pulmonary vascular remodeling and cor pulmonale in rats with monocrotaline-induced PH and in mice with chronic hypoxia-induced PH [8]. Perros et al. reported that PDGF-BB-induced proliferation and migration of PASMCs from patients with IPAH were inhibited by imatinib [10].

Not only inhibition of proliferation but also induction of apoptosis of PASMCs is needed to actively reduce stenosis due to vascular remodeling at small pulmonary arteries of patients with IPAH. These two effects may lead to reverse remodeling of the pulmonary vasculature. Expression of PDGF-B is up-regulated in the medial layer of small pulmonary arteries of rats with monocrotaline-induced PH and imatinib induces apoptosis in the small pulmonary arteries [8]. However, imatinib does not induce apoptosis in cultured IPAH-PASMCs without PDGF treatment [10]. Thus, imatinib may not be able to induce apoptosis in quiescent cells. We hypothesized that imatinib in the presence of PDGF-BB induces apoptosis of PASMCs from patients with IPAH, but that imatinib cannot induce apoptosis in PASMCs without PDGF stimulation. We therefore investigated whether imatinib in the presence and absence of PDGF-BB induces apoptosis of PASMCs from patients with IPAH.

Akt is a member of the serine/threonine-specific kinase family known for facilitating cell survival via the inhibition of apoptotic pathways [12]. Therefore, induction of apoptosis of IPAH-PASMCs may be related to Akt inactivation. We also investigated whether imatinib inhibits Akt activation.

2. Materials and methods

2.1. Isolation, culture and identification of PASMCs

Peripheral segments of the pulmonary artery were obtained at lung transplantation [13] from 8 patients with IPAH as previously described [5,6,14,15] (2 males and 6 females; mean age, 22 ± 4 years; age range 11–43 years) (Table 1). For normal control experiments, samples of pulmonary arteries were also obtained at lung lobectomy from a patient with bronchogenic carcinoma (male, 58 years old) who showed no evidence of PAH and received no systemic chemotherapy or radiation therapy before lung lobectomy as previously described [5,6,14,15]. Samples of the pulmonary arteries were obtained from the most distal area from the carcinoma in the resected lobe. All of the studies were approved by the Ethics Committee of Okayama University Graduate School of Medicine, Density, and Pharmaceutical Sciences, and written informed consent was obtained from all patients before the procedure. The investigation also conforms to the principles outlined in the Declaration of Helsinki.

PASMCs were isolated as described previously [5,6,14–16]. Peripheral segments of pulmonary arteries smaller than 1 mm in outer diameter were disaggregated with collagenase and cut into 2-mm-long sections, and then the adventitia and endothelial cell layers were removed. Vessels were plated on a 6-well plate with Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Sigma) and 0.1 mg/mL kanamycin (Sigma) and incubated in a humidified 5% CO2 atmosphere at 37 °C. The culture medium was changed every 3 days. After reaching confluence, the cells were subcultured by treatment with trypsin

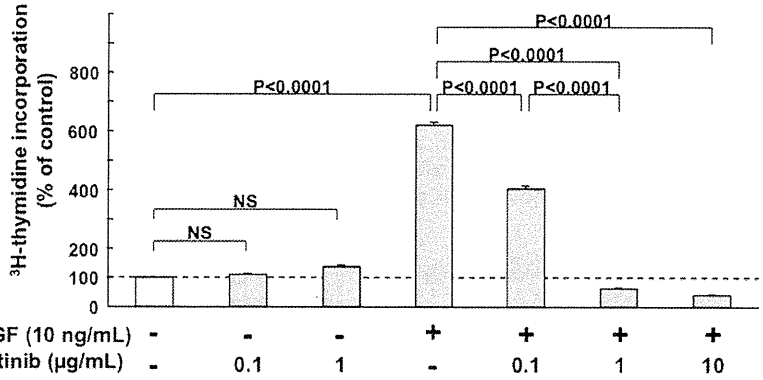

Fig. 1. Inhibitory effect of imatinib on proliferation of PASMCs from IPAH patients. Anti-proliferative effects of imatinib (0.1 to 10 μg/mL) on IPAH-PASMCs stimulated with PDGF-BB (10 ng/mL). ^{3}H-thymidine incorporation was measured. Counts per minute (cpm) were expressed as a percentage of cpm of IPAH-PASMCs treated with a diluent (control). Data are mean ± SE.

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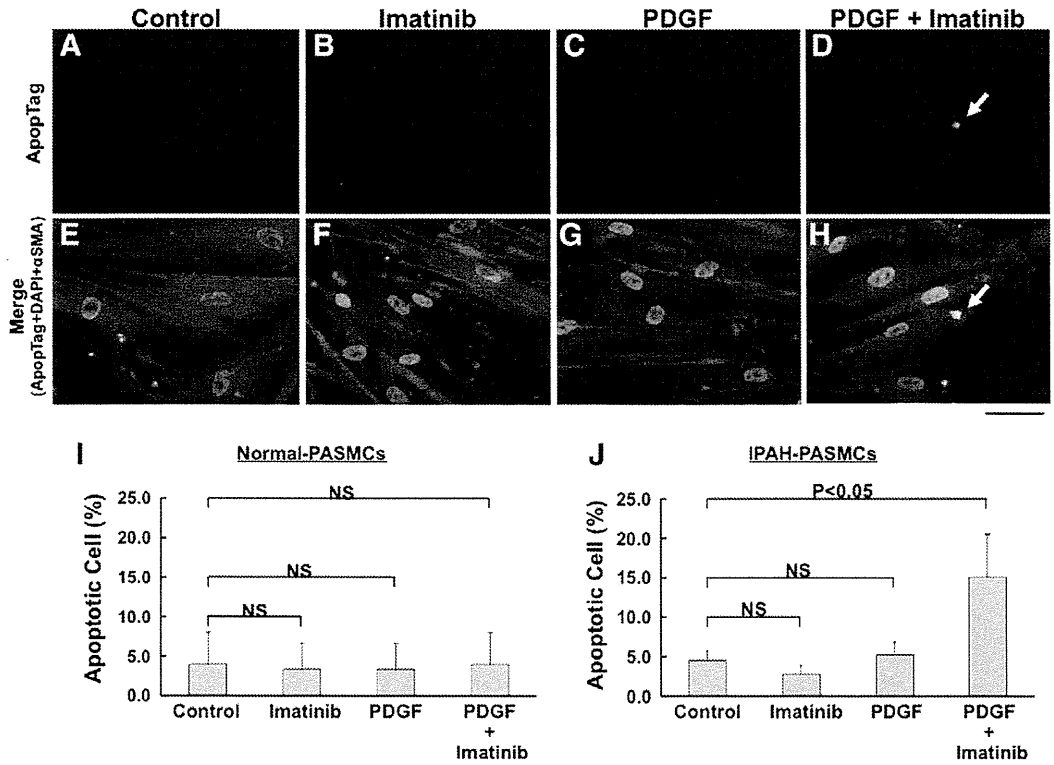

Fig. 2. Effect of imatinib on apoptosis of PASMCs in TUNEL assay by ApopTag fluorescein. A to D, ApopTag fluorescein (green). E to H, Combined images (merge) of ApopTag fluorescein, DAPI (blue) and αSMA (red). A and E, IPAH-PASMCs without treatment. B and F, IPAH-PASMCs treated with imatinib (1 μg/mL). C and G, IPAH-PASMCs treated with PDGF-BB (10 ng/mL). D and H, IPAH-PASMCs treated with imatinib and PDGF-BB. Arrow shows a TUNEL-positive cell (green). Bar = 500 μm. I, Effect of imatinib on apoptosis of normal PASMCs in TUNEL assay. J, Effect of imatinib on apoptosis of IPAH-PASMCs in TUNEL assay. Imatinib (1 μg/mL) in the presence of PDGF-BB (10 ng/mL) significantly increased TUNEL-positive (apoptotic) cells in IPAH-PASMCs compared with the control condition ($P<0.05$). Data are mean ± SE.

(0.05%)/ethylenediaminetetraacetic acid (EDTA) (0.02%). Cell identification was confirmed by the examination of cytoskeletal components (α-smooth muscle actin, myosin, and smoothelin) using an immunocytochemical technique as described previously [5,15]. Cells between passages 3 to 5 were used for all experiments.

2.2. Effects of imatinib on cell proliferation

To assess the antiproliferative effect of imatinib on PASMCs, we measured ^{3}H-thymidine incorporation using methods described previously [5,16]. PASMCs were reseeded in 24-well plates at a density of 5×10^4 cells/well on day 0. After 16 h of incubation (on day 1), the culture media were replaced with low-serum culture media (DMEM, 0.1% FBS, and 0.1 mg/mL kanamycin), and the cultured cells were made quiescent for 48 h. On day 3, PDGF-BB (10 ng/mL) (Sigma), imatinib (0.1 to 10 μg/mL) (Novartis) or an Akt inhibitor, Akt-I-1/2 (1 μmol/L) (Calbiochem), was added to the media. After 21 h (on day 4), the cells were labeled with ^{3}H-thymidine at 1 μCi/mL for 3 h. After completion of labeling, the cells were washed with ice-cold PBS, fixed with 5% trichloroacetic acid and 95% ethanol, and lysed with 200 μL/well of 0.33 mol/L NaOH. Aliquots of the cell lysates were neutralized with 1 mol/L HCl, and the radioactivity was measured in a liquid scintillation analyzer (TRI-CARB 2200CA; Packard, Downers Grove, IL, USA).

2.3. Western blot analysis

PASMCs from patients with IPAH were prepared in the same manner as that described for analysis of DNA synthesis. They were treated in the presence or absence of PDGF-BB (10 or 100 ng/mL), imatinib (1 or 10 μg/mL) and a mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK) inhibitor, U0126 (3 μmol/L) (Promega). Western blot analysis was performed as described previously [5,7]. Briefly, total cell lysates of cultured PASMCs were extracted in commonly used radioimmunoprecipitation (RIPA) buffer with 10 mg/mL phenylmethylsulfonyl fluoride (Sigma) and then concentrated by centrifugation at 12,000 rpm for 20 min. Protein samples (10 μg) were loaded on 10% sodium dodecyl sulfate-polyacrylamide gel and blotted onto nitrocellulose membranes. Blots were incubated with rabbit anti-p27 antibody (Santa Cruz Biotechnology), anti-GAPDH antibody (Chemicon), anti-phospho-Akt antibody and anti-total-Akt antibody (Cell Signaling Technology Inc., Beverly, MA). The relative integrated density of each protein band was digitized by NIH image J 1.34 s.

2.4. Evaluation of apoptosis

TUNEL assays were performed using an ApopTag fluorescein in situ apoptosis detection kit (Chemicon International Inc.) according to the manufacturer's instructions as described previously [17]. Nuclear morphology was examined by labeling with DAPI solution (0.6 μg/mL, Dojindo Laboratories). Immunofluorescence staining was performed to confirm α-smooth muscle actin (αSMA) expression using αSMA antibody (1:100 dilution, Sigma). Caspase assay was performed using a CaspaTag Caspase-3/7 in situ apoptosis detection kit (Chemicon International Inc.) according to the manufacturer's instructions. Nuclear morphology was examined by Hoechst staining. The samples were analyzed by fluorescence microscopy (Olympus IX71, Olympus Optical Co. Ltd, Tokyo, Japan). For each cover slip, 5–10 fields (with 10–30 cells in each field) were randomly selected to determine the percentage of apoptotic cells in total cells based on the morphological characteristics of apoptosis. PASMCs were reseeded on collagen-coated glass cover slips in 12-well plates at a density of 5×10^4 cells/well on day 0. After 16 h of incubation (on day 1), the culture media were replaced with low-serum culture media (DMEM, 0.1% FBS, and 0.1 mg/mL kanamycin), and the cultured cells were made quiescent for 48 h. On day 3, PDGF-BB (10 ng/mL), imatinib (1 μg/mL) or Akt-I-1/2 (1 μmol/L) was added to the media. After 24 h (on day 4), the cells were stained by using an ApopTag fluorescein in situ apoptosis detection kit or CaspaTag in situ apoptosis detection kit.

Transmission electron microscopy was performed with an electron microscope (H-7100: Hitachi; Tokyo, Japan).

To observe cellular apoptosis with a time-lapse system (Olympus Optical Co.), PASMCs were cultured on a 35-mm culture dish that has a micro-photolithographed squared pattern (Kuraray Co., Ltd., Tsukuba, Japan) [7] so that the apoptotic cells will not disappear from view.

2.5. Statistical analysis

All results are expressed as mean ± SE. Statistical significance for comparison between the two measurements was determined using Student's *t* test. For comparison between the different treatment groups, statistical analysis was performed using one-

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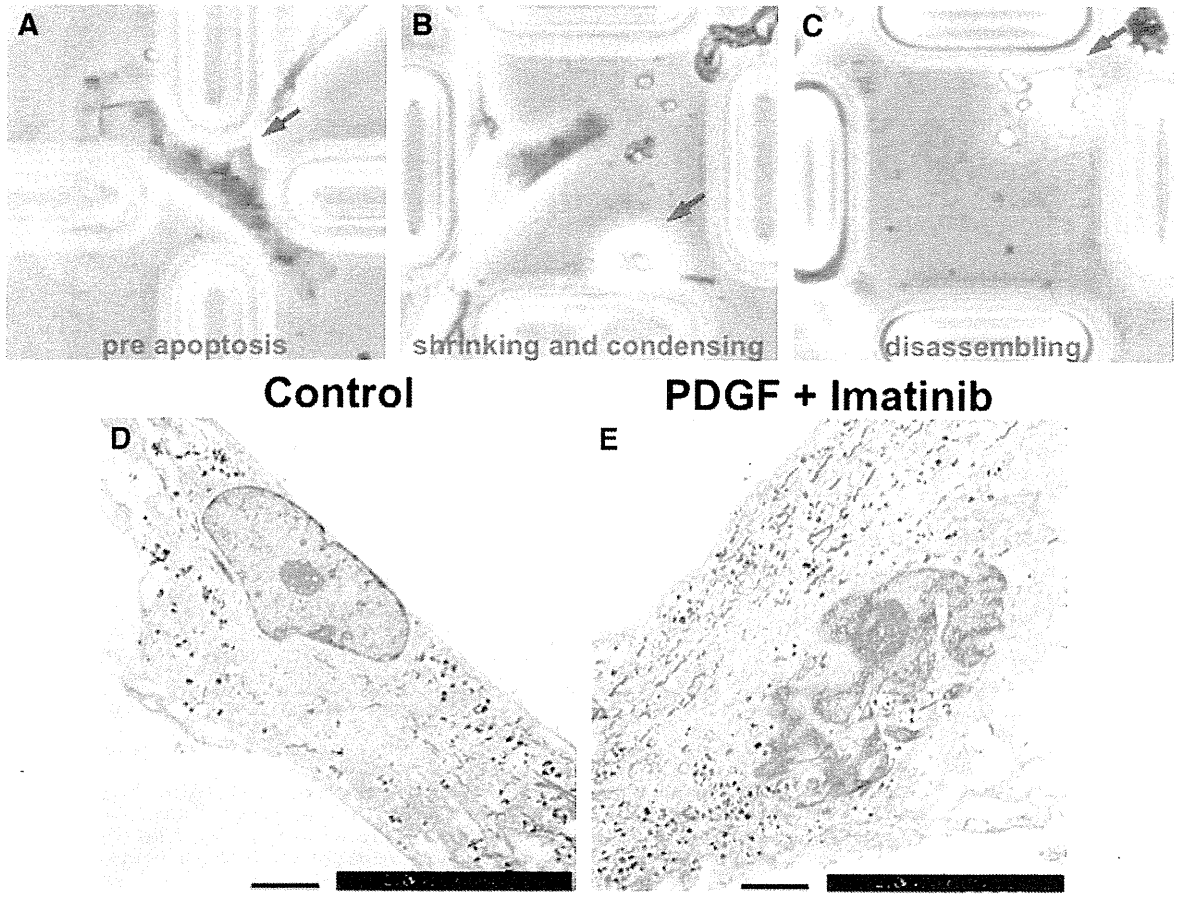

Fig. 3. Effect of imatinib on apoptosis of PASMCs in time-lapse microscopy and transmission electron microscopy. A to C, Representative images of time-lapse microscopy. IPAH-PASMCs were treated with imatinib (1 μg/mL) and PDGF-BB (10 ng/mL). Bar = 20 μm. D and E, Representative images of transmission electron microscopy. D, IPAH-PASMCs without treatment (control). E, IPAH-PASMCs treated with imatinib (1 μg/mL) and PDGF-BB (10 ng/mL). Bar = 5 μm.

way ANOVA with Fisher's PLSD test. Values of $P<0.05$ were considered to be statistically significant.

3. Results

3.1. Inhibitory effect of imatinib on proliferation of PASMCs from IPAH patients

Treatment with imatinib inhibited PDGF-BB-induced proliferation of PASMCs from IPAH patients as assessed by ^{3}H-thymidine incorporation (n = 5–12 experiments in each cell) (Fig. 1). This result is consistent with recent findings of other investigators [10].

3.2. Effect of imatinib on apoptosis of PASMCs from IPAH patients

We performed a TUNEL assay using an ApopTag fluorescein to assess the effect of imatinib on apoptosis of PASMCs from IPAH patients. Fig. 2 shows representative cases of the TUNEL assay. TUNEL-positive cell (green) was observed after 24-hour treatment with imatinib (1 μg/mL) in the presence of PDGF-BB (10 ng/mL) (Fig. 2D and H). However, imatinib (1 μg/mL) (Fig. 2B and F) or PDGF-BB (10 ng/mL) (Fig. 2C and G) alone did not induce apoptosis in IPAH-PASMCs. Imatinib (1 μg/mL) in the presence of PDGF-BB (10 ng/mL) significantly increased TUNEL-positive cells in IPAH-PASMCs compared with the control condition in IPAH-PASMCs ($P<0.05$: 15.1 ± 5.4% versus 4.5 ± 1.3%, n = 4 or 5 experiments in each cell line) (Fig. 2J). There was no significant difference in the percentage of TUNEL-positive cells between the imatinib alone or PDGF alone condition and the control condition (Fig. 2J). There was also no significant difference between the imatinib alone, PDGF alone or both imatinib and PDGF condition and the control condition in normal PASMCs (P = NS, n = 5 experiments) (Fig. 2I).

Fig. 3A, B and C shows the apoptosis induced by the combination of imatinib (1 μg/mL) and PDGF-BB (10 ng/mL) in IPAH-PASMCs as assessed by time-lapse microscopy. A PASMC shows shrinking and condensing and finally disassembling. Fig. 3E shows a transmission electron microscopic image of an apoptotic cell in IPAH-PASMCs. Condensation of chromatin along the nuclear membrane and fragmentation of the nucleus were observed in cultured IPAH-PASMCs treated with imatinib (1 μg/mL) and PDGF-BB (10 ng/mL).

Fig. 4 shows representative cases of the caspase assay in IPAH-PASMCs. Caspase-3 and −7-active cell was observed after 24-hour treatment with imatinib (1 μg/mL) in the presence of PDGF-BB (10 ng/mL) (Fig. 4D and H). Imatinib (1 μg/mL) in the presence of PDGF-BB (10 ng/mL) significantly increased caspase-3 and −7-active cells in IPAH-PASMCs compared with the control condition ($P<0.01$: 12.4 ± 3.0% versus 2.2 ± 1.2%, n = 5 experiments in each cell line) (Fig. 4J). There was no significant difference in the percentage of caspase-3 and −7-positive cells between the imatinib alone or PDGF alone condition and the control condition in IPAH-PASMCs (Fig. 4J). There was also no significant difference between the imatinib alone, PDGF alone or both imatinib and PDGF condition and the control condition in normal PASMCs (P = NS, n = 5 experiments) (Fig. 3I).

These results show that imatinib did not induce apoptosis in normal PASMCs and quiescent IPAH-PASMCs but that imatinib had a proapoptotic effect on IPAH-PASMCs stimulated with PDGF.

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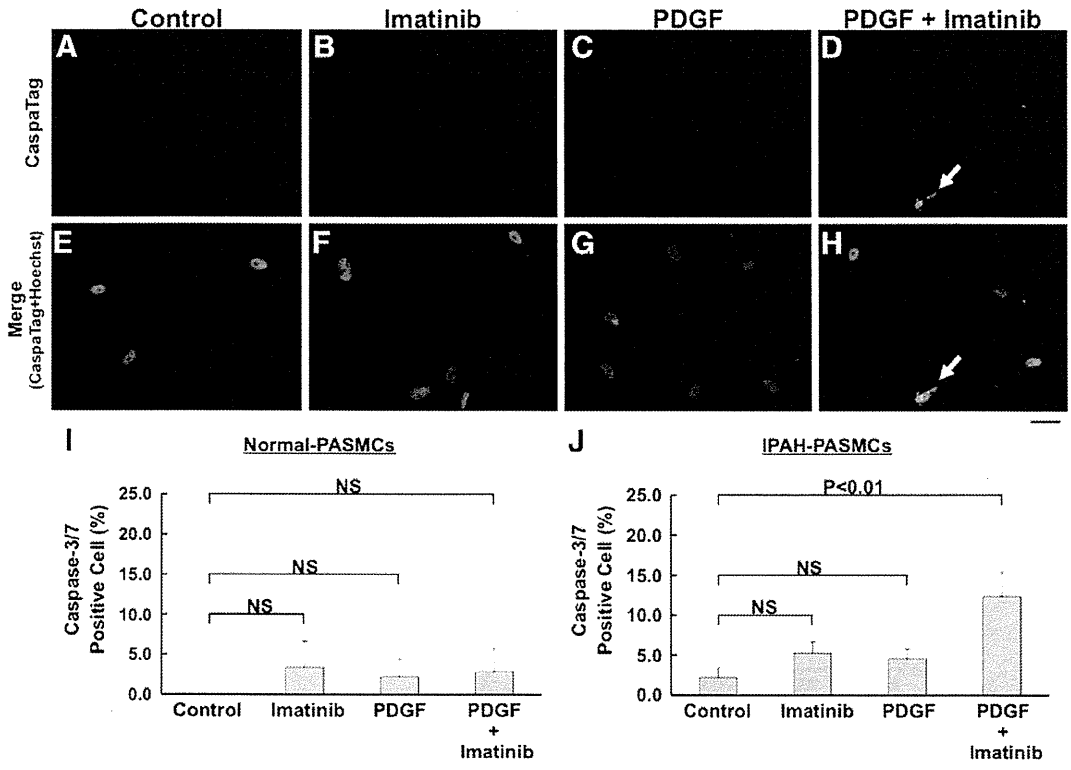

Fig. 4. Effect of imatinib on apoptosis of PASMCs in Caspase assay using a CaspaTag Caspase-3/7 in situ apoptosis detection kit. A to D, CaspaTag staining (green). E to H, Combined images (merge) of CaspaTag staining and Hoechst nuclear staining (blue). A and E, IPAH-PASMCs without treatment. B and F, IPAH-PASMCs treated with imatinib (1 μg/mL). C and G, IPAH-PASMCs treated with PDGF-BB (10 ng/mL). D and H, IPAH-PASMCs treated with imatinib and PDGF-BB. Arrow shows a caspase-3/7-positive cell (green). Bar = 500 μm. I, Effect of imatinib on apoptosis of normal PASMCs in Caspase assay. J, Effect of imatinib on apoptosis of IPAH-PASMCs in Caspase assay. Imatinib (1 μg/mL) in the presence of PDGF-BB (10 ng/mL) significantly increased caspase-positive (apoptotic) cells in IPAH-PASMCs compared with the control condition ($P<0.01$). Data are mean ± SE.

3.3. Effect of imatinib on PDGF-BB-induced phosphorylation of Akt

Western blot analysis revealed that PDGF-BB induced phosphorylation of Akt at 15 min (Fig. 5A, lanes 2 and B). Akt phosphorylation was significantly inhibited by imatinib (1 ng/mL) compared with the treatment with PDGF-BB ($P<0.05$, $n=4$ experiments) (Fig. 5A, lanes 3 and B).

Akt-I-1/2 (1 μmol/L), an Akt inhibitor, could mimic the effects of imatinib on PASMCs. Akt-I-1/2 significantly inhibited PDGF-induced proliferation of IPAH-PASMCs as assessed by ^{3}H-thymidine incorporation ($P<0.001$, $n=10$ experiments) (Fig. 5C). Akt-I-1/2 in the presence of PDGF-BB significantly increased TUNEL-positive cells ($P<0.05$, $n=5$ experiments) (Fig. 5D) and caspase-3,7-positive cells in IPAH-PASMCs ($P<0.05$, $n=5$ experiments) (Fig. 5E) compared with the control condition. These results show that the inhibition of Akt is strongly related to the anti-proliferative and pro-apoptotic effects of imatinib on PDGF-stimulated IPAH-PASMCs.

4. Discussion

Two major new findings were obtained in the present study. First, imatinib did not induce apoptosis in quiescent IPAH-PASMCs and normal PASMCs, but it had a pro-apoptotic effect on IPAH-PASMCs stimulated with PDGF. Second, inhibition of Akt is related to the anti-proliferative and pro-apoptotic effects of imatinib on PDGF-stimulated IPAH-PASMCs.

Imatinib alone did not induce apoptosis in IPAH-PASMCs. This result is consistent with recent findings of other investigators [10]. However, the combination of imatinib and PDGF induced apoptosis. Therefore, imatinib did not induce apoptosis in quiescent IPAH-PASMCs, but it had a pro-apoptotic effect on IPAH-PASMCs stimulated with PDGF. It has been reported that PDGF-A and PDGF-B mRNA levels were increased in small pulmonary arteries from patients with IPAH [10] and that serum PDGF-BB levels across the lung circulation were higher in IPAH patients [18]. Therefore, imatinib is expected to induce apoptosis in clinical settings. Further studies are needed to clarify this point.

Many signaling pathways, including ERK, p38 MAPK and Akt, are involved in proliferation and survival of PASMCs [14,19]. Akt is a member of the serine/threonine-specific kinase family known for facilitating cell survival via the inhibition of apoptotic pathways. It has been shown that PDGF stimulation transiently phosphorylates Akt and the mammalian target of rapamycin (mTOR) in PASMCs from patients with chronic thromboembolic pulmonary hypertension [19]. In our study, PDGF-BB induced phosphorylation of Akt and it was inhibited by imatinib in IPAH-PASMCs. Akt-I-1/2, an Akt inhibitor, could mimic the effects of imatinib on PASMCs. Akt is related to the anti-proliferative and pro-apoptotic effects of imatinib on PDGF-stimulated IPAH-PASMCs.

Imatinib is a drug used for treating chronic myelogenous leukemia and gastrointestinal stromal tumors. However, resistance to imatinib can occur [20–22]. Not only primary resistance within the first two months but also secondary resistance develops after a median of about 2 years of treatment with the drug. Hatano et al. reported that imatinib decreases the plasma concentration of PDGF-BB in patients with PAH, while the improvement in hemodynamic parameters is transient [11]. We showed that imatinib had a pro-apoptotic effect on IPAH-PASMCs stimulated with PDGF in the present study. Thus, imatinib would induce apoptosis only in the early period of treatment when plasma PDGF-BB levels are relatively high. After the PDGF levels have decreased, imatinib would not be able to induce apoptosis. Therefore, resistance to imatinib might occur in patients with pulmonary hypertension. Attention is needed in clinical use.

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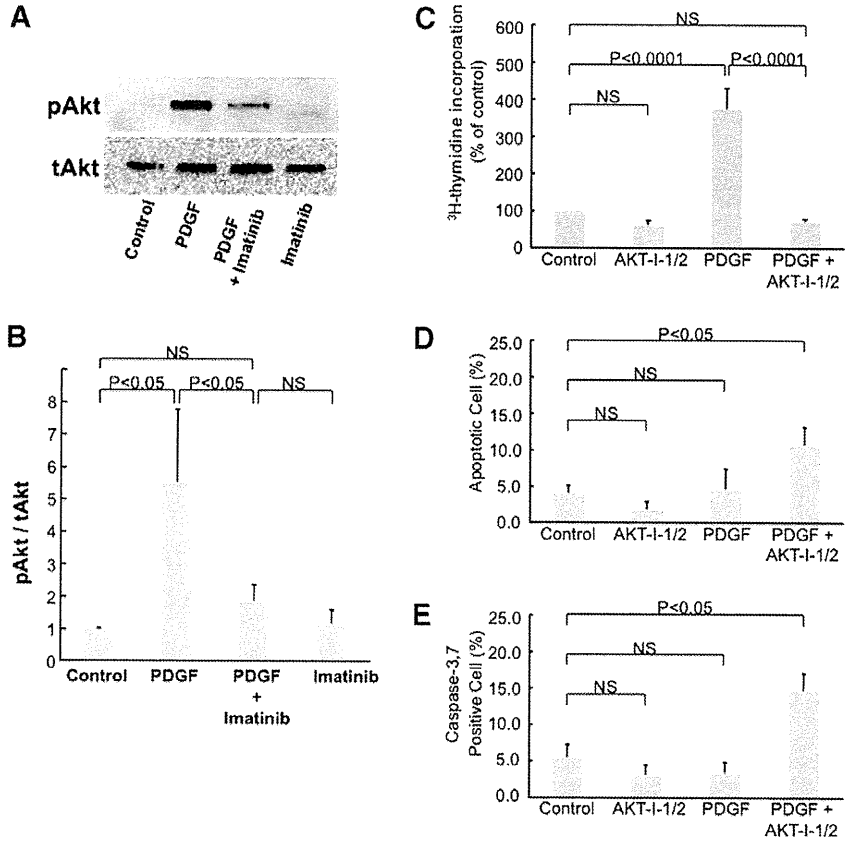

Fig. 5. Effect of imatinib on PDGF-BB-induced phosphorylation of Akt and effects of an Akt inhibitor on PDGF-BB-stimulated proliferation and apoptosis of PASMCs. A, Western blot analysis of total Akt (tAkt) and phosphorylated Akt (pAkt). PDGF-BB (10 ng/mL) induced phosphorylation of Akt at 15 min (lanes 2). Akt phosphorylation was inhibited by imatinib (1 ng/mL) (lanes 3). B, Bar graphs show semiquantitive analysis of pAkt expression level in IPAH-PASMCs. Data are mean ± SE of the intensity of the band corresponding to pAkt relative to tAkt. C, Anti-proliferative effect of Akt-I-1/2 (1 μmol/L), an Akt inhibitor, on IPAH-PASMCs stimulated with PDGF-BB (10 ng/mL). ^{3}H-thymidine incorporation was measured. Counts per minute (cpm) were expressed as a percentage of cpm of IPAH-PASMCs treated with a diluent (control). Data are mean ± SE. D, Effect of Akt-I-1/2 (1 μmol/L) on apoptosis of PASMCs in TUNEL assay by ApopTag fluorescein. E, Effect of Akt-I-1/2 (1 μmol/L) on apoptosis of PASMCs in Caspase assay using a CaspaTag Caspase-3/7 in situ apoptosis detection kit.

In conclusion, imatinib inhibited PDGF-induced proliferation of IPAH-PASMCs. Imatinib did not induce apoptosis in quiescent IPAH-PASMCs, but it had a pro-apoptotic effect on IPAH-PASMCs stimulated with PDGF. Inhibition of Akt may be important in the anti-proliferative and pro-apoptotic effects of imatinib on PDGF-stimulated IPAH-PASMCs. Modulation of PDGF signaling such as Akt is important. Inhibition of PDGF signaling by imatinib may become a useful molecular-targeted therapy for IPAH.

Conflict of interest

There are no relationships with industry.

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Circulating KCNH2 Current-Activating Factor in Patients with Heart Failure and Ventricular Tachyarrhythmia

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Abstract

Background: It is estimated that approximately half of the deaths in patients with HF are sudden and that the most likely causes of sudden death are lethal ventricular tachyarrhythmias such as ventricular tachycardia (VT) or fibrillation (VF). However, the precise mechanism of ventricular tachyarrhythmias remains unknown. The KCNH2 channel conducting the delayed rectifier K^+ current (I_{Kr}) is recognized as the most susceptible channel in acquired long QT syndrome. Recent findings have revealed that not only suppression but also enhancement of I_{Kr} increase vulnerability to major arrhythmic events, as seen in short QT syndrome. Therefore, we investigated the existence of a circulating KCNH2 current-modifying factor in patients with HF.

Methodology/Principal Findings: We examined the effects of serum of HF patients on recombinant I_{Kr} recorded from HEK 293 cells stably expressing KCNH2 by using the whole-cell patch-clamp technique. Study subjects were 14 patients with non-ischemic HF and 6 normal controls. Seven patients had a history of documented ventricular tachyarrhythmias (VT: 7 and VF: 1). Overnight treatment with 2% serum obtained from HF patients with ventricular arrhythmia resulted in a significant enhancement in the peaks of I_{Kr} tail currents compared to the serum from normal controls and HF patients without ventricular arrhythmia.

Conclusions/Significance: Here we provide the first evidence for the presence of a circulating KCNH2 channel activator in patients with HF and ventricular tachyarrhythmias. This factor may be responsible for arhythmogenesis in patients with HF.

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Introduction

Heart failure (HF) remains a major clinical problem all over the world.[1] It is estimated that approximately half of the deaths in these patients is sudden and that the most likely causes of sudden cardiac death (SCD) are lethal cardiac arrhythmia such as ventricular tachycardia (VT) or fibrillation (VF).[2] It has been known for a long time that the failing heart undergoes a complex electrical remodeling of ventricular myocytes and that a consequent reduction of repolarization reserve and electrical instability may predispose to an increased risk of life-threatening arrhythmia.[3,4]

KCNH2, also called human ether-a-go-go-related gene (hERG) potassium channel, is responsible for the rapid components of delayed rectifier potassium currents (I_{Kr}). I_{Kr} is a major contributor to the repolarization process of cardiac action potentials and is recognized as the most susceptible channel in acquired long QT syndrome.[5] Recent findings have revealed that not only suppression but also enhancement of I_{Kr} increase vulnerability to major arrhythmic events. A gain-of-function mutation in KCNH2 has also gained recognition as a congenital disorder characterized by a higher risk for major arrhythmic events and SCD, referred to as short QT syndrome (SQT).[6,7] It has also been reported that PD-118057, an I_{Kr}-activating agent, predisposes to cardiac arrhythmias *in vitro*.[8] HF also induces remodeling of I_{Kr}, but there is no widespread consensus on suppression or enhancement of I_{Kr} in previous studies.[9,10] Furthermore, despite the incremental progress in understanding intrinsic I_{Kr} modulators, to our knowledge, there has been no report of naturally occurring substances with a KCNH2 current-activating effect in patients with HF. Therefore, we investigated the existence of a circulating KCNH2 current-activating factor in patients with HF.

Results

Characteristics of the study subjects

Demographics and selected clinical characteristics of the HF patients are summarized in Table 1. The etiology of HF in the

study population consisted of DCM (n = 8), documented myocoarditis (n = 3), dilated hypertrophic cardiomyopathy (n = 1), tachycardia-induced cardiomyopathy (n = 1) and apical ballooning syndrome (n = 1). Ventricular tachyarrhythmias were documented in 8 patients: non-sustained VT in 4 (50%), sustained VT in 3 (38%) and VF in 1 (13%). There were no significant differences in the mean age between the control group, the HF without VT and VF (VT/VF (−)) group, and the HF with VT and VF (VT/VF (+)) group (42.7±14.8 yrs, 50.7±10.9 yrs and 53.3±10.8 yrs, respectively; $P = 0.28$). Male-to-female ratio was also not significantly different between the groups (2:1, 2:1 and 1:1 for the control, VT/VF (−) and VT/VF (+) groups, respectively; $P = 0.76$). Mean QTc interval was longer in the overall HF patients than in the controls (0.46±0.03 $sec^{1/2}$ vs. 0.42 ± 0.03 $sec^{1/2}$; $P<0.05$), but there was no significant difference between the VT/VF (−) and VT/VF (+) groups (0.45±0.03 $sec^{1/2}$ vs. 0.47±0.02 $sec^{1/2}$; $P = 0.51$). Mean left ventricular ejection fraction (EF) was similar in the VT/VF (−) and VT/VF (+) groups (39±11% vs. 31±13%, $P = 0.22$).

More than half of the patients in the VT/VF (+) group took cardiotonic agents, whereas none of the patients in the VT/VF (−) group took cardiotonic agents (Table 2). However, none of the drugs, including cardiotonic agents, taken by the subjects have been reported to activate KCNH2 current.

Enhancement of KCNH2 currents by serum from HF patients with ventricular tachyarrhythmia

We investigated the effect of 2% serum of the study subjects on recombinant I_{Kr} recorded from HEK293 cells stably expressing KCNH2. KCNH2 tail currents were similar in the control and VT/VF (−) group but were significantly increased in the VT/VF (+) group ($P<0.05$: VT/VF (+) vs. control and VT/VF (−)) (Figure 1). There was no significant effect on voltage dependency of KCNH2 activation in these groups (Table 3). There was no relationship between peak tail amplitude and EF in the overall HF patients (Figure 2).

These results indicate the presence of a circulating KCNH2 channel activator in patients with HF and ventricular tachyarrhythmias.

Efficacy of class III antiarrhythmic agents

Case No.1 was treated with amiodarone, and Case No.6 was treated with d-sotalol. Both drugs, which exert their antiarrhythmic actions essentially by blocking I_{Kr},were effective in controlling VT and VF.

Discussion

The major new finding of this work is that the factor causing activation of the KCNH2 current is present in serum of patients with HF accompanied by cardiac arrhythmia. Although there are several reports on suppression of I_{Kr} by circulating hormones or auto-antibodies, this is the first report providing evidence for a naturally occurring I_{Kr} activator.[5,11,12] I_{Kr} conducted by KCNH2 channels is the major repolarizing outward current of ventricular action potential, and it has been shown to have a strong association with life-threatening arrhythmia in many pathological circumstances. Our finding indicates that enhancement of I_{Kr} can play a key role in arrhythmogenesis in the setting of HF.

The majority of research efforts have focused classically on reduction of I_{Kr} as an approach for understanding arrhtymogenesis because it is well recognized that reduction of I_{kr} means decrease in net repolarizing current that results in prolongation of action potential duration (APD) and QT interval in the ECG and development of early afterdepolarization-induced triggered activity following torsade de pointes (TdP).[13] On the other hand, much attention has been paid to upregulation of KCNH2 in recent years because of its causal relationship to cardiac arrhythmias. SQT is a recently recognized clinical concept characterized by short QT intervals in the ECG, and it is associated with major cardiac events leading to SCD without organic heart disease.[7] The first identified form of SQT resulted from gain-of-function mutation in KCNH2. Mutation N588K in KCNH2 causes an increase in net repolarizing current.[6] In addition, recent studies have revealed a significant association between early repolarization and SQT in clinical phenotype.[14,15] Because of the high prevalence of early repolarization detected in SQT, it is thought that enhancement of repolarization is a common mechanism underlying arryhthmogenicity in early

Table 1. Clinical characteristics of the study population.

Case No.	Age/Sex	Diagnosis	NYHA	LVEF (%)	Arrhythmia	QTc ($sec^{1/2}$)
1	73/F	myocarditis	II	50	sustained VT	0.46
2	45/M	DCM	II	11	NSVT	0.47
3	55/F	dilated-HCM	IV	25	NSVT	0.47
4	37/F	DCM	III	19	sustained VT	0.48
5	61/F	DCM	II	37	NSVT	0.43
6	52/M	myocarditis	II	42	VF	0.45
7	48/M	Tachycardia-induced cardiomyopathy	II	24	NSVT	0.48
8	64/F	Apical ballooning syndrome	II	47	none	0.43
9	61/F	myocarditis	II	42	none	0.48
10	45/M	DCM	II	50	none	0.39
11	55/M	DCM	III	40	sustained VT	0.51
12	37/M	DCM	II	40	none	0.47
13	55/M	DCM	II	38	none	0.47
14	42/M	DCM	II	20	none	0.46

NYHA: New York Heart Association Functional Classification, LVEF: left ventricular ejection fraction, DCM: idiopathic dilated cardiomyopathy, HCM: hypertrophic cardiomyopathy, VT: ventricular tachycardia, NSVT: non-sustained ventricular tachycardia, VF: ventricular fibrillation, QTc: corrected QT interval

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Table 2. Patient's medication.

Case No.	Medications						
	Antiarrhythmics	Beta- blockers	Diuretics	ACEI/ARB	Anticoagulant/ Antiplatelet	Cardiotonic	others
1	amiodarone	carvedilol	torasemide	candesartan	none	none	none
2	none	carvedilol	furosemide spironolactone	valsartan	none	pimobendan	nateglinide, acarbose
3	none	carvedilol	furosemide spironolactone	imidapril	none	dopamine dobutamine milrinone	lansoprazole
4	amiodarone	carvedilol	torasemide	losartan	warfarin	dopamine dobutamine	none
5	none	carvedilol	furosemide	candesartan	warfarin	none	alfacalcidol
6	sotalol	carvedilol	none	candesartan	warfarin	none	potassium chloride, famotidine
7	none	carvedilol	furosemide spironolactone	valsartan	warfarin	metildigoxin	lansoprazole
8	none	none	none	losartan	aspirin	none	nifedipine, famotidine, atorvastatin, icosapentate
9	none	carvedilol	furosemide spironolactone	losartan	warfarin	none	rabeprazole, ferrous citrate
10	none	none	none	none	none	none	none
11	none	carvedilol	torasemide trichlormethiazide	candesartan	warfarin	metildigoxin	pravastatin
12	none	carvedilol	none	candesartan	none	none	amlodipine
13	none	carvedilol	furosemide spironolactone	candesartan	warfarin aspirin	none	none
14	none	carvedilol	furosemide	telmisartan	warfarin	none	amlodipine

ACEI/ARB: Angiotensin-Converting Enzyme Inhibitors/Angiotensin Receptor Blockers.
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repolarization and SQT.[14] Since QT interval is affected by other ion channels and cardiac enlargement, QT interval of HF patients with ventricular tachyarrhythmia in this study was not short. However, the circulating KCNH2 channel activator in this study is potentially responsible for electrical instability.

Effects of KCNH2-activating agents have also been evaluated. It has been reported that PD-118057, established as a selective KCNH2 current enhancer without affecting activation, confers inducibility of both ventricular tachyarrhythmia and atrial fibrillation.[8,16] The functional similarity between this agent and circulating KCNH2-activating factor in having no significant effect on the voltage dependence of activation suggests a causal relationship of this intrinsic enhancer with arrhythmogenesis in HF. In addition, this concept is endorsed by the efficacy of class III antiarrhythmic agents for treatment of ventricular tachyarrhythmia in the study subjects. Since the dominant action of class III antiarrhythmic agents is to lengthen the APD by a reduction of I_{Kr}, it is thought that the therapeutic effect of the agents against arrhythmia in the study subjects is manifested by offsetting oversupplied KCNH2 currents.

On the other hand, there are only a few reports on the antiarrhythmic potential of an I_{Kr} activator in specialized pathologic settings. PD-118057 prevented the early after-depolarization induced by class III antiarrhythmic agents, and NS3623 decreased the frequency of bradycardia-induced extrasystoles *in vitro*.[17,18] However, these data were obtained only in constrained experimental conditions and couldn't refer clinical benefit for arrhythmogenic disorders such as long QT syndrome.

The mechanism by which exposure of ion channels to this factor activates the KCNH2 current remains to be elucidated. It is also uncertain whether the KCNH2-activating effect is the cause or consequence of HF-induced remodeling of action potential. However, exertion of their functional effects may be involved in arrhythmogenesis in HF. Further studies are needed to clarify these points.

In conclusion, our data show that a novel KCNH2 activator exists in serum of HF patients with ventricular tachyarrhythmia and is potentially responsible for electrical instability.

Methods

All of the studies were approved by the Ethics Committee of Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, and written informed consent was obtained from all patients before the procedure. The investigation also conforms to the principles outlined in the Declaration of Helsinki.

Study population

Serum samples were obtained from 14 HF patients referred to Okayama University Hospital, Okayama, Japan between December 2007 and December 2008 who consented to undergo evaluation of the pathogenesis of HF. HF was defined as the presence of current or previous symptoms of exercise intolerance and EF of 50% and less with no other cause of exercise intolerance. Exclusion criteria included coronary heart disease, primary valvular heart disease, severe systemic disease, or severe pulmonary disease. The mean age of the patients was 52 ± 10 years, and 8 patients (57%) were male. Control sera were obtained from 6 subjects without cardiovascular disease or abnormal ECG

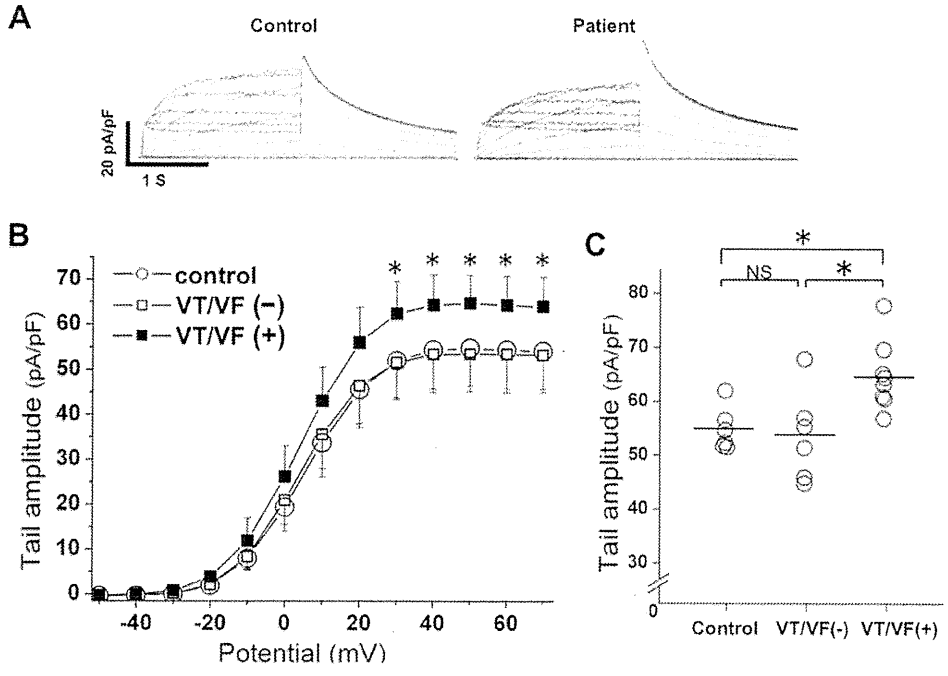

Figure 1. Effect of serum obtained from HF patients with ventricular tachyarrhythmia on KCNH2 currents in HEK 293 cells. A. Representative traces from a single cell cultured in a medium supplemented with 2% serum of a control subject (left) and medium supplemented with 2% serum of a patient with sustained VT (Case No. 1) (right). B. Current-voltage relationships of KCNH2 tail currents. Mean values of peak KCNH2 tail current densities were calculated by averaging the tail amplitude of each subject obtained from cells (n = 8 to 11 cells) exposed to respective sera. Open circles show the results for controls (n = 6), open squares show the results for HF patients without VT and VF (VT/VF (−), n = 6) and closed squares show the results for HF patients with VT/VF (VT/VF (+), n = 8). *P<0.05: VT/VF (+) vs. control and VT/VF (−). C. Maximum values of peak tail current in the groups. *P<0.05: VT/VF (+) vs. control and VT/VF (−). NS indicates not significant.
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findings. Mean age of the control subjects was 43±15 years, and 4 subjects (67%) were male.

Patch-clamp recordings

KCNH2 channel currents were recorded at room temperature (22±1°C) by using the whole-cell patch-clamp technique as previously described.[11] The control bath solution contained (mM): 132 NaCl, 4.8 KCl, 1.2 $MgCl_2$, 2 $CaCl_2$, 5 glucose, 10 Hepes, pH 7.4. Pipettes (2–4 MΩ resistances) were filled with a pipette solution containing (mM): 110 potassium aspartate, 5 K2-ATP, 11 EGTA, 5 Hepes, 1 $CaCl_2$, and 1 $MgCl_2$, pH 7.3. Currents were normalized to cell capacitance to give the measure of current density. There was no difference in membrane capacitance between the groups (14.1±2.0 pF, 14.2±2.8 pF, and 14.7±1.6 pF for the control, VT/VF (−) and VT/VF (+) groups, respectively; P = 0.88). KCNH2 channel tail-current amplitude was monitored at 0.1 Hz by analysis of peak deactivating tail current recorded at −40 mV after 2-s depolarizing test pulses to +20 mV from a holding potential (V_H) of −80 mV. Current zero level (no activation) was determined by applying 25-ms pulses to −40 mV preceding the test pulses. HEK293 cells stably expressing KCNH2 were cultured for 1 day

Table 3. Changes in voltage dependence of the KCNH2 activation.

	Controls	VT/VF(−)	VT/VF(+)	P
$V_{1/2}$ (mV)	5.6±4.8	3.7±3.5	4.3±2.5	NS
Slope factor (k)	8.2±0.8	8.2±0.9	8.6±1.0	NS

VT/VF (−): HF patients without VT and VF, VT/VF (+): HF patients with VT and VF
NS indicates not significant.
doi:10.1371/journal.pone.0019897.t003

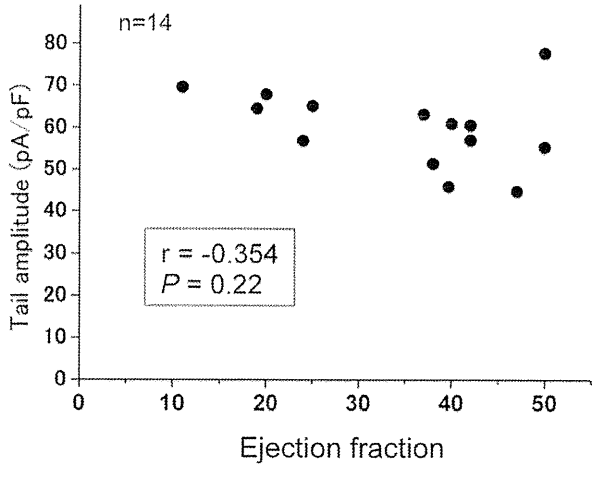

Figure 2. Relationship between peak tail amplitude and ejection fraction in the overall HF patients.
doi:10.1371/journal.pone.0019897.g002

in a medium to which 2% serum obtained from the study subjects had been added. The KCNH2 current activation was studied by analysis of deactivating tail currents recorded at −40 mV after a series of 2-s test pulses from −50 mV to +70 mV (10 mV increments; V_H, −80 mV). Pulse frequency was 0.1 Hz. Peaks of tail currents were plotted as function of the KCNH2 activation. Activation curves were analyzed with a fit of each data to the Boltzmann equation, $I/I_{max} = G/G_{max} = \{1+\exp[-(V_m - V_{1/2})/k]\}^{-1}$. G/G_{max} is normalized chord conductance at V_m to the maximum chord conductance. $V_{1/2}$ is the potential where the conductance is half-maximally activated, and k is the slope factor.

ECG measurements

Twelve-lead ECGs were recorded to measure the parameters of repolarization. The QT interval was measured from the start of the QRS complex to the end of the T wave, defined as the return to the iso-electric baseline. They were corrected to heart rate using Bazett's formula: QTc (QT/$\sqrt{RR}$).

Data Analysis

All values are presented as means±.D. Statistical significance was assessed with ANOVA followed by post hoc Scheffe's method. A value of $P<.05$ was considered statistically significant. pCLAMP 9.2 software (Axon Instruments) was used to both acquire and analyze data for the patch-clamp experiments. Graphical analyses were carried out using Origin 7.0 J software (Microcal). Statistical analysis was performed with StatView 5.0 (Abacus Concepts, Inc., Berkeley, CA, U.S.A.).

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Author Contributions

Conceived and designed the experiments: KN. Performed the experiments: HS KN HM SA YT YK JK TF. Analyzed the data: KN NN SN KK HM TM KFK HI. Contributed reagents/materials/analysis tools: JK TF. Wrote the paper: HS KN. Supervisor: TO HI.

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CARDIO VASCULAR DIABETOLOGY

ORIGINAL INVESTIGATION Open Access

Association of increased plasma adipocyte fatty acid-binding protein with coronary artery disease in non-elderly men

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Abstract

Background: Adipocyte fatty acid-binding protein (A-FABP) has been reported to play critical roles in the development of atherosclerosis. We investigated whether an increased in plasma A-FABP level can be independently associated with the presence of coronary artery disease (CAD).

Methods: Two hundred eleven consecutive male patients (mean age: 66 years, range: 33-87 years) were enrolled from inpatients who underwent coronary angiography. Age-matched male subjects (n = 211) having no evidence of CAD served as controls. Plasma A-FABP levels were measured by enzyme-linked immunosorbent assays.

Results: Plasma A-FABP levels in CAD patients were significantly higher than in control subjects (median [IQR], 20.6 [15.7-27.8] ng/mL vs. 15.1 [11.7-19.9] ng/mL, $p < 0.01$). Multivariate logistic regression analysis revealed that an increased plasma A-FABP level was independently associated with the presence of CAD in all subjects (adjusted odds ratio: 1.76, 95% confidence interval: 1.14 to 2.70, $p = 0.01$). Furthermore, sub-analysis based on age showed that this association remained significant in subjects aged < 65 years (adjusted odds ratio: 3.06, 95% confidence interval: 1.34 to 6.98, $p < 0.01$), but not in subjects aged ≥65 years.

Conclusions: Increased plasma A-FABP in non-elderly men had a significant association with the presence of CAD, independent of established CAD risk factors.

Keywords: adipocyte, fatty acid-binding protein, coronary artery disease, risk factor

Introduction

Adipocyte fatty acid-binding protein (A-FABP), also known as aP2 or FABP4, is a small intracellular lipid-binding protein [1]. There are nine types of FABPs, showing tissue-specific expression patterns, and A-FABP is abundantly expressed in adipocytes and macrophages [2]. Similar to other FABPs, recent studies showed that A-FABP plays an essential regulatory role in energy metabolism and inflammation [1]. The pathophysiological role of this molecule has been investigated in murine experimental models. A-FABP-deficient mice were protected from the development of insulin resistance in diet-induced obesity[3], type 2 diabetes [4], and atherosclerosis in models of hypercholesterolemia [5]. A-FABP had an effect on atherosclerosis due to not only the dysregulation of systemic metabolism related with adipose tissue, as a result of the activation of macrophages, as it was reported that the expression of A-FABP in macrophages is induced by oxidized low-density lipoprotein (LDL) [6], but also Toll-like receptor activators [7]. It was also reported that an inhibitor of A-FABP markedly reduced atherosclerotic lesions in an ApoE-/- mouse model [5].

Clinically, the involvement of A-FABP in atherosclerosis is supported by a genetic study in human subjects. A carrier of T-87 C polymorphism had lower serum triglyceride levels, demonstrating a reduced cardiovascular risk [8]. Moreover, although A-FABP was originally a cytoplasmic protein, A-FABP levels could be detected in human serum [9]. Higher serum A-FABP has been reported to be useful for the prediction and diagnosis of

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