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Effects of the Endothelin Receptor Antagonist Bosentan on Hemodynamics, Symptoms and Functional Capacity in Japanese Patients With Severe Pulmonary Hypertension

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Background Endothelin (ET)-1 has a pathogenic role in pulmonary arterial hypertension (PAH). Recent clinical studies carried out in Western populations showed that blockade of the ET receptors by bosentan improves pulmonary hemodynamics and exercise capacity. In the present study, the efficacy of bosentan was assessed in Japanese patients with PAH.

Method and Results Because the pharmacokinetics of bosentan and its metabolites are similar in Japanese and Caucasian subjects, the same dose of bosentan, 125 mg twice daily, was administered in the Japanese open-label clinical trial. In 18 patients, mean pulmonary arterial pressure decreased from 52.4±13.8 to 46.8±13.8 mmHg (p=0.003) and cardiac index increased from 2.20±0.74 to 2.61±0.72 $L \cdot min^{-1} \cdot m^{-2}$ (p=0.002). The 6-min walking distance increased from 410±89.5 to 494±86.0 m (p<0.0001). The dyspnea index (Borg scale) decreased from 3.2±2.4 to 2.2±1.7 (p=0.02). The specific activity scale (SAS) gradually increased throughout the study period from 2.9±0.8 to 4.6±1.9 METs (p=0.0005). WHO Class improved in 10 patients.

Conclusion Bosentan was well tolerated and improved the hemodynamics, symptoms, exercise capacity, and quality of life of Japanese patients with PAH. Thus, bosentan can be a valuable therapeutic option in Japanese patients. (*Circ J* 2005; **69:** 131–137)

Key Words: Bosentan; Endothelin receptor antagonist; Pulmonary arterial hypertension; Quality of life

Pulmonary arterial hypertension (PAH) is a rare and debilitating disease, characterized by an increase in pulmonary vascular resistance that ultimately leads to right heart failure and death[1] When a definite cause can not be demonstrated, the condition is termed primary pulmonary hypertension (PPH), which predominantly affects women most commonly in their third decade of life[2] No ethnic predisposition is apparent in the National Institutes of Health registry, and the proportions by ethnic group parallel those in the general population[2] Similar pulmonary vascular lesions are produced by many illnesses such as scleroderma, human immunodeficiency virus infection, liver disease or the use of certain anorectic drugs, and these are now classified as types of PAH[3] A limited number of innovative strategies for the treatment of PAH have been developed over the past decades, but their effectiveness is largely limited by their nonselectivity for the pulmonary vasculature and significant drawbacks have been reported[5]

Recently, it was shown that PAH is associated with increased concentrations of endothelin (ET)-1, a potent vasoconstrictor, in plasma and the lungs[6,7] suggesting that inhibition of ET receptors is a potential therapeutic alternative for this life-threatening disorder. In fact, studies with Caucasian PAH patients have demonstrated significant clinical benefits of bosentan, a dual ET receptor antagonist[8–10] In the present study, the effects of bosentan on cardiopulmonary hemodynamics, symptoms and functional capacity were assessed, as well as the 6-min walk test and the specific activity scale (SAS), in Japanese patients with PAH.

The pharmacokinetics of bosentan are dose-proportional up to a dose of 500 mg and in Caucasians, the absolute bioavailability of bosentan is 50%, being mainly excreted via the bile in the form of metabolites[11,12] However, ethnic differences in the pharmacokinetics of many drugs have been demonstrated[13] Therefore, prior to the start of the clinical trial, the multiple-dose pharmacokinetics of bosentan were compared in Caucasian and Japanese subjects.

Methods

Comparative Study of the Ethnic Differences in the Pharmacokinetics of Bosentan

This part of the study was performed at FOCUS GmbH

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(Neuss, Germany). The protocol was approved by the independent Ethics Committee of the "Aerztekammer Nordrhein" (Düsseldorf, Germany). All subjects gave written informed consent before any screening procedures were performed. Six male and 7 female healthy Caucasians (age 23–49 years) and 6 male and 7 female Japanese subjects (age 21–45 years) were assigned to treatment with 125 mg b.i.d. of bosentan for 7.5 days. Although the pharmacokinetics of bosentan are not influenced by food,[11,12] the meals were standardized and Japanese subjects received typical Japanese food and European food was served to the Caucasian subjects throughout the study period.

Blood samples of 4 ml were obtained immediately before drug administration in the morning of days 2–8 and at several time points (every hour for the first 6 h, every 2 h for the subsequent 10 h and finally after 24 h) after drug administration on day 8. Plasma was separated and stored at –20°C pending analysis. The concentration of bosentan and its active metabolite, Ro 48-5033 were determined by a liquid chromatography method with tandem mass spectrometry detection.[14] The limit of quantification was 1.0 ng/ml for bosentan and 2.0 ng/l for Ro48-5033.

The pharmacokinetic evaluation for bosentan and Ro48-5033 used model independent methods.[15] The peak plasma concentration (C_{max}) and the time to C_{max} (t_{max}) were read directly from the concentration–time data. The area under the plasma concentration–time curve (AUC) was estimated by the linear trapezoidal rule and log-linear regression analysis of the terminal phase. Pharmacokinetic parameters were analyzed descriptively, calculating the geometric mean and 95% confidence intervals or for t_{max}, the median and range.

The study was powered to detect with 90% power a difference of 50% in AUC_τ between the 2 ethnic groups. Differences between Caucasian and Japanese subjects for the bosentan and metabolite pharmacokinetic parameters were explored using the 2-sample t-test on logarithmically transformed C_{max}, AUC_τ, and $t_{1/2}$ values, and the 2-sample Wilcoxon test for t_{max}.

Clinical Study of the Effects of Bosentan in Japanese Patients With PAH

Japanese patients aged over 20 years were eligible for enrollment in the study if they (1) had symptomatic, severe PPH or PAH because of connective-tissue disease (scleroderma or systemic lupus erythematosus (SLE)), (2) were in functional classes III–IV according to the 1998 World Health Organization (WHO) classification despite conventional therapy, (3) met the following hemodynamic criteria within 2 months of enrollment: mean pulmonary arterial pressure (mPAP) >25 mmHg at rest, pulmonary capillary wedge pressure (PCWP) <15 mmHg, and pulmonary vascular resistance (PVR) >240 dyn·s/cm^5, (4) had a baseline 6-min walk test between 150 and 500 m. Patients were excluded if they were pregnant, had hypotension (systolic blood pressure <100 mmHg), hypokalemia or other significant systemic disease. The institutional ethics review committees approved the protocol and written informed consent was obtained from all patients.

At baseline, within 2 months prior to the start of treatment, hemodynamic measurements were performed with a Swan-Ganz catheter while patients were recumbent. Cardiac output (CO) was obtained by the thermodilution method using the mean of 3 measurements. The cardiac index (CI) was derived by normalization of CO with the body surface area (BSA) (CI=CO/BSA). PVR was calculated from the transpulmonary gradient and CO (PVR=[mPAP–PCWP]/CO). The patients' symptoms were evaluated by the Borg dyspnea index (a measure of perceived breathlessness on a scale of 0–10, with higher values indicating more severe dyspnea)[16] and the WHO functional class for pulmonary hypertension. Efficacy of treatment was also assessed by the 6-min walk test and the specific activity scale (SAS).[17] To determine the SAS, patients were asked to specify the extent of physical activity they could perform without symptomatic limitation. Summarizing these data, the patient was categorized by the metabolic costs expended with the most strenuous possible activity.

After the baseline assessments, bosentan (Tracleer, Actelion, Allschwil, Switzerland) was started at a dose of 62.5 mg once daily for the first week, then 62.5 mg twice daily for the next 3 weeks, and finally 125 mg twice daily for the subsequent 8 weeks. Hemodynamic measurements were performed after the 12 weeks of treatment. Symptoms, physical examinations, electrocardiogram, 6-min walk test, WHO classification, and SAS were assessed every 4 weeks. Safety was assessed on the basis of recorded adverse events, clinical laboratory parameters, vital signs, and electrocardiography.

Statistical Analysis

The PVR as the primary efficacy parameter, and other hemodynamic values at week 12 were compared with the baseline on a per protocol population basis by using the signed-ranks test as primary analysis. A significant change was defined as $p<0.05$ (two-tailed). In a patient in whom bosentan treatment was terminated because of worsening of the disease, the hemodynamic data obtained at the last observation were used for analysis. If data were not available, the imputation rule of using the worst data (pre-treatment value in this case) was used. If the data at 12 weeks were not available because of termination of the treatment for other reasons, the last data between 8 and 12 weeks were adopted for analysis. The missing values for other measurements were excluded from the analysis. To confirm the robustness of the results, sensitive analysis based on the ITT (intention to treat) was used.

Results

Comparative Study of the Ethnic Differences in the Pharmacokinetics of Bosentan

Twenty-six subjects participated in the study and 24 completed the entire study in accordance with the protocol. Two subjects prematurely withdrew because of adverse events: myalgia of moderate intensity in 1 female Caucasian and a first-degree atrioventricular block in 1 Japanese female subject. Therefore, 26 subjects were evaluated for safety and 24 for pharmacokinetics.

Steady-state concentrations of bosentan were attained after 5–6 days of administration in both ethnic groups (data not shown). The mean plasma concentration–time curves and pharmacokinetic parameters of bosentan and its metabolite, Ro48-5033, are presented in Fig 1 and Table 1. The 2-sample t-test did not yield any statistically significant differences between the 2 ethnic groups.

Of the 47 adverse events that occurred during the study, 19 were reported by Caucasian and 28 by Japanese subjects. Headache of mild to moderate intensity was the most frequently reported adverse event in both ethnic

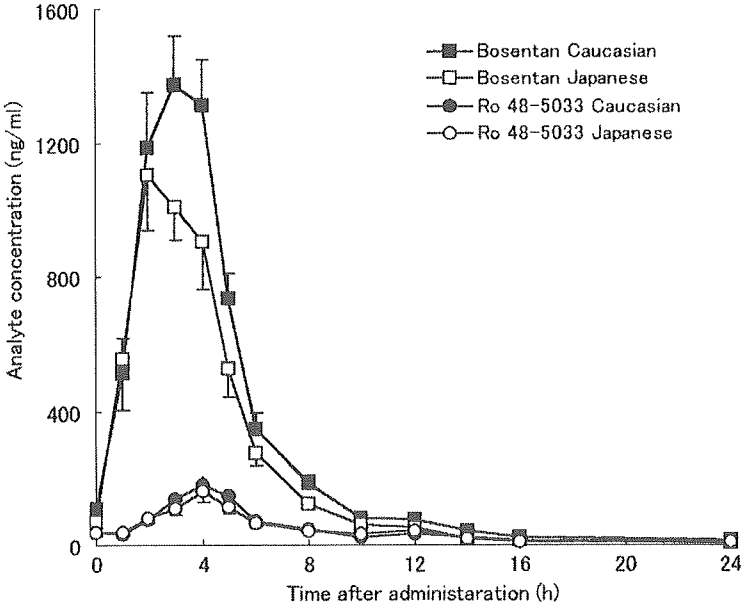

Fig 1. Mean plasma concentration–time curves of bosentan and its metabolite, Ro48-5033 in 12 healthy Caucasian and Japanese subjects after administration of 125 mg of bosentan. Data are presented as arithmetic means ± SEM.

Table 1 Pharmacokinetic Parameters of Bosentan and Its Metabolite in Caucasian and Japanese Subjects After Administration of 125 mg of Bosentan

Group	C_{max} *(ng/ml)*	t_{max} *(h)*	*AUC (ng·h/ml)*	$t_{1/2}$
Bosentan				
Caucasian	*1,434*	*3.5*	*6,046*	*7*
	(1,137, 1,808)	*(2.0, 4.0)*	*–49,997,311*	*(5.3, 9.3)*
Japanese	*1,212*	*3*	*4,640*	*5.6*
	(940, 1,564)	*(1.0, 4.0)*	*(3,641, 5,914)*	*(4.6, 6.9)*
Ro 48-5033				
Caucasian	*175*	*4*	*859*	*10.6*
	(138, 221)	*(3.0, 5.0)*	*(3,641, 5,914)*	*(4.6, 6.9)*
Japanese	*136*	*4*	*721*	*9.6*
	(92, 201)	*(3.9, 5.0)*	*(532, 977)*	*(7.7, 11.8)*

Data are expressed as geometric mean (95% confidence limits).
AUC, area under curve; C_{max}, peak plasma concentration; t_{max}, time to C_{max}.

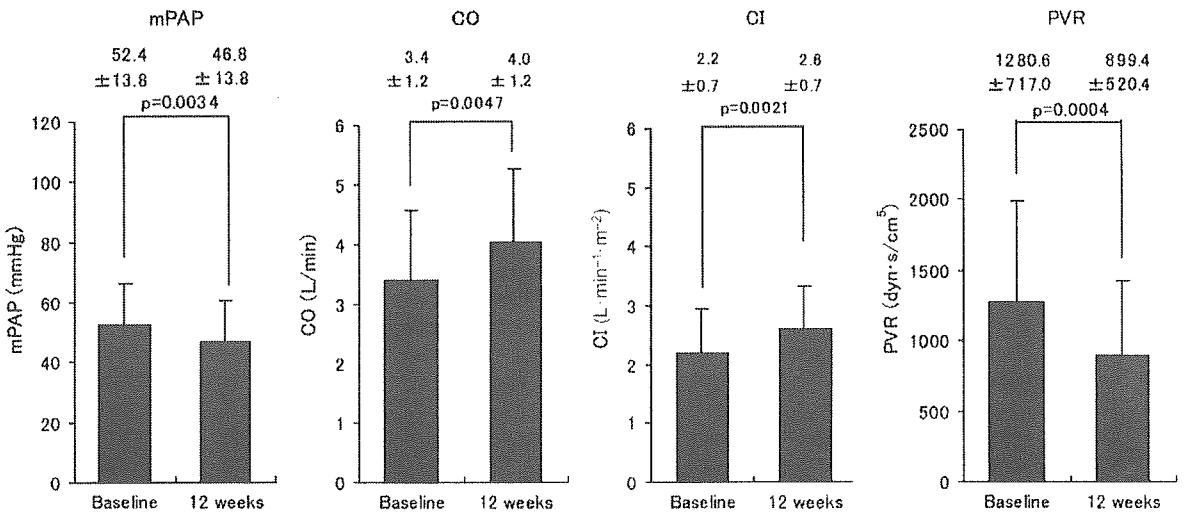

Fig 2. Effect of bosentan on hemodynamic parameters from baseline to week 12 (mean ± SEM). mPAP, mean pulmonary arterial pressure; CO, cardiac output; CI, cardiac index; PVR, pulmonary vascular resistance.

Table 2 Changes in the Hemodynamic Parameters After 12-Week Treatment Program With Bosentan 125 mg b.i.d. in 18 Patients With Severe Pulmonary Hypertension

Hemodynamic parameters	*Baseline*	*Week 12*	*p value*	*ITT**
Systolic pulmonary arterial pressure (mmHg)	*85.9±23.6*	*76.7±23.7*	*0.0106*	*0.0074*
Diastolic pulmonary arterial pressure (mmHg)	*33.1±8.6*	*28.3±9.1*	*0.0147*	*0.0182*
Mean pulmonary arterial pressure (mmHg)	*52.4±13.8*	*46.8±13.8*	*0.0034*	*0.0030*
Pulmonary capillary wedge pressure (mmHg)	*6.3±2.7*	*7.8±3.4*	*0.0309*	*0.0297*
Cardiac output (L/min)	*3.39±1.19*	*4.02±1.22*	*0.0047*	*0.0192*
Cardiac index ($L \cdot min^{-1} \cdot m^{-2}$)	*2.20±0.74*	*2.61±0.72*	*0.0021*	*0.0135*
Pulmonary vascular resistance ($dyn \cdot s/cm^5$)	*1,281±717*	*899±520*	*0.0004*	*0.0003*
Right arterial pressure (mmHg)	*4.9±4.0*	*5.4±3.7*	*0.3134*	*0.3510*

**Sensitive analysis by intension-to-treat.*

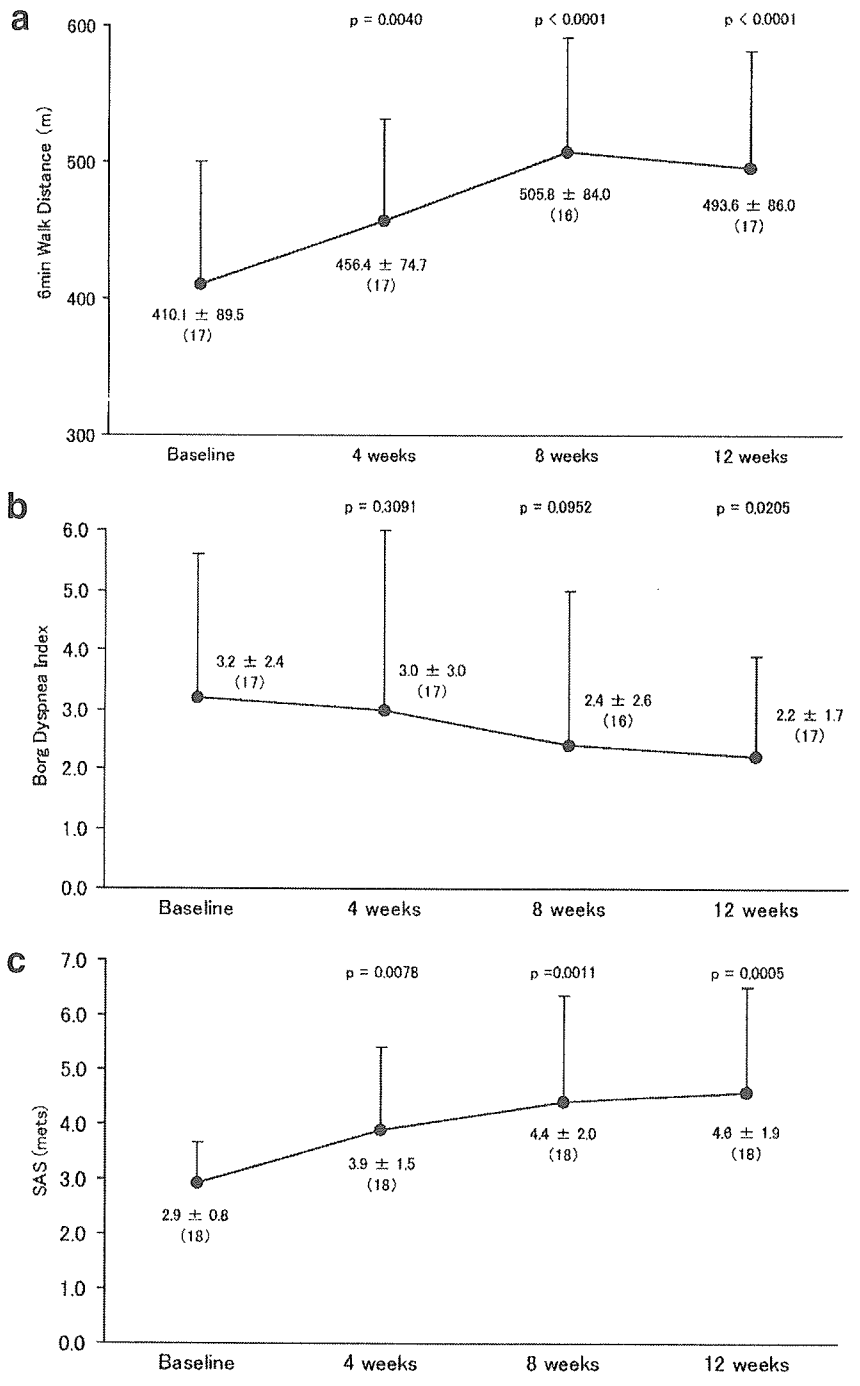

Fig 3. (a) Change in the 6-min walking distance from baseline to week 12. (b) Change in the Borg dyspnea scale from baseline to week 12. (c) Change in the specific activity scale (SAS) from baseline to week 12. Data are expressed as mean± SEM and numbers in parentheses indicate the number of patients assessed.

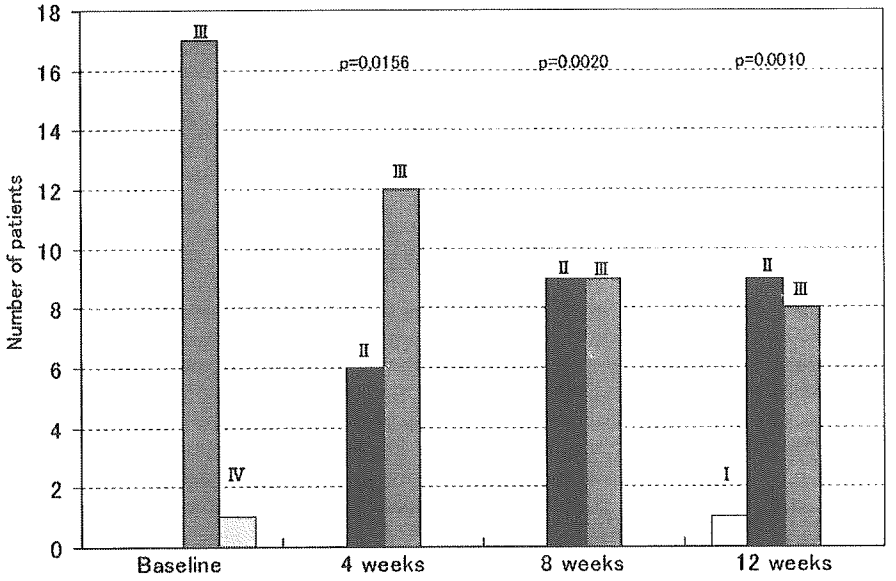

Fig 4. Change in World Health Organization (WHO) functional class from baseline to week 12.

groups. Following administration of bosentan, there were slight and transient decreases in systolic and diastolic blood pressure (5–7 mmHg) but these changes did not appear to be clinically significant. At the end-of-study examination, 4 of 13 Japanese subjects and 2 of 13 Caucasian subjects had alanine aminotransferase (ALAT) values above the upper limit of normal, defined as 23 and 19 U/L in male and female subjects, respectively. The absolute values of ALAT did not exceed 67 U/L in any subject and the increases were not considered clinically significant.

Clinical Study of the Effects of Bosentan in Japanese Patients With PAH

Twenty-one patients were recruited from 11 centers. One patient was excluded from the analysis of efficacy because hemodynamic data at week 12 were not available and another 2 patients were excluded because of technical problems that precluded an accurate measurement of hemodynamic parameters. Therefore, 18 patients (2 males, 16 females), 13 with PPH and 5 with PAH (4 secondary to SLE and 1 secondary to mixed connective tissue disease) were finally included in the analysis of efficacy and 21 were assessed for safety. The mean age was 36±10 years (range, 21–60 years).

After 12 weeks of treatment with bosentan, PVR decreased from 1,281±717 to 899±520 dyn·s/cm^5 ($p<0.0004$). Improvements in other hemodynamic parameters were also observed; for example, mPAP reduced from 52.4±13.8 to 46.8±13.8 mmHg ($p<0.0034$), and CI increased from 2.20±0.74 to 2.61±0.72 L·min^{-1}·m^{-2} ($p<0.0021$) (Fig 2, Table 2). Systolic blood pressure was reduced from 113.0±13.3 to 106.6±9.7 mmHg and diastolic blood pressure from 72.7±11.6 to 66.2±5.2 mmHg, but neither of these changes reached statistical significance. No cases of clinically significant hypotension were observed during the study.

After 12 weeks of treatment, the distance walked in 6 min increased by 83.5±64.1 m ($p<0.0001$) (Fig 3a) and the changes in the Borg dyspnea index paralleled the improvements observed in the 6-min walk test; that is, the index decreased gradually from 3.2±2.4 to 2.2±1.7 throughout the study period, but the changes reached statistical significance only at week 12 ($p=0.0205$) (Fig 3b). The SAS values averaged 2.9±0.8 METs at baseline and increased continuously and significantly, reaching 4.6±1.9 METs at the final assessment ($p=0.0005$) (Fig 3–3). At the beginning of the study, 17 patients were in WHO Class III and 1 in Class IV, but by the end of the study 10 patients had improved to Class I or II ($p=0.0010$) (Fig 4), leaving 8 patients in Class III.

Bosentan, at a dose of 125 mg twice daily, was well tolerated. Adverse drug reactions (excluding unrelated) were observed in 14 of 21 patients (66.7%), including headache (38.1%), dizziness (19.0%), and myalgia (14.3%). Abnormal values of laboratory tests were noted in 10 patients. Bosentan treatment was associated with an increase in aspartate aminotransferase and ALAT (38.1%), an increase in bilirubin (14.3%), a decrease in hemoglobin (14.3%) and a decrease in leukocytes (14.3%). Of 8 patients who had increases in liver aminotransferase concentrations, 3 had concentrations more than 3-fold the upper limit of normal, necessitating discontinuation of the study medication in 1 case. In the other 2 cases, the aminotransferase concentrations returned to normal without discontinuation of treatment, continuing either at the same dose or at a reduced dose of 62.5 mg twice daily. In the other 5 cases, aminotransferase concentrations did not increase more than twice the upper limit of the normal range and returned to the normal range by the end of the study in 4 cases without dose adaptation.

Discussion

Pulmonary arterial hypertension is rapidly progressive, leading to right heart failure and death in a median of 2.8 years from diagnosis. For the majority of cases, the treatments so far developed have been only palliative and the limited oral treatment options include long-term anticoagulant therapy and therapy with calcium-channel blockers, prostacyclin analogues, or phosphodiesterase inhibitors.[19,20] The introduction of intravenous epoprostenol in 1990s greatly improved survival, but this treatment is expensive, the dosage required to sustain these effects increases with time, adverse effects are frequent because of pump malfunction, catheter-related infections and thrombosis, or the

drug induces significant side effects. The efficacy of epoprostenol analogues that can be inhaled (iloprost) or administered orally (beraprost) remains to be confirmed.[21]

It has been recently suggested that local production of ET-1 plays a pathogenic role in PAH, as evidenced by its high plasma concentrations in patients with PPH or PAH,[7,22] the increased expression of ET-1 in the lungs of patients with pulmonary hypertension[6] or idiopathic pulmonary fibrosis.[23] Endothelin-1 has 2 receptors, A and B. Activation of ETA receptors produces vasoconstriction and smooth muscle growth, whereas activation of ETB receptors induces nitric oxide production and vasodilation. Therefore, development of an ET-receptor blocker specific for ETA appears to be desirable. On the other hand, because the ETB receptor mediates release of aldosterone from the adrenal cortex,[24] nonselective blockade of both ETA and ETB receptors may have additional benefits by inhibiting collagen synthesis. Bosentan is an orally effective, nonselective antagonist of ETA and ETB receptors and recent clinical trials have documented promising results in patients with severe pulmonary hypertension[9,10,18] although its effects are yet to be well characterized in Japanese subjects.

Numerous clinical studies have shown that ethnic groups may differ in their responsiveness to drugs,[25–27] and it has also been suggested that racial differences in the catalytic activity of cytochrome P450 (CYP) isozyme may be responsible for the differences in drug kinetics.[28] The International Conference on Harmonization guideline (ICHE5) document "Ethnic Factors in the Acceptability of Foreign Data" recommends the measurement of pharmacokinetic/pharmacodynamic parameters to permit the clinical effects obtained in one population to be extrapolated to a different population.[13] Ethnic differences in the drug pharmacokinetics depend on gut metabolism/transport and most commonly on hepatic first pass metabolism, but the ethnic differences in hepatic metabolism are known to be unpredictable by race and specific enzyme.[13] The present study showed that the pharmacokinetics of bosentan at the dose of 125 mg are similar in Caucasian and Japanese subjects. Bosentan is metabolized by CYP2C9 and CYP3A4 to 3 metabolites and excreted in bile.[29] A study that used healthy volunteers from broadly defined ethnic groups to assess the adenine to guanine transition in the 5' promoter region of the CYP3A4 gene in a sequence motif known as the nifedipine-specific element, indicated considerable racial differences in the frequency of this polymorphism between Caucasian and Japanese subjects, but there was no ethnic difference in the rate of CYP3A4-dependent drug metabolism and this promoter region polymorphism was considered not to play a major role in determining constitutive CYP3A4 expression.[30] When differences in CYP3A activity between Caucasian and Japanese subjects were assessed using midazolam as an in vivo probe, no statistically significant or clinically important interracial/ethnic difference was observed.[31] Therefore, we assumed that no dose adjustment was necessary when bosentan was used to treat Japanese patients and conducted the first open-label clinical trial of bosentan at the same dose as used in the previous studies carried out in Western populations.

This study demonstrated that 12 weeks of treatment with bosentan at a dose of 125 mg twice daily resulted in significant improvement in symptoms as measured by Borg dyspnea index, exercise capacity as assessed by the 6-min walk test and the SAS, together with an improvement in hemodynamic parameters. The changes in the 6-min walking distance and Borg dyspnea index indicated that patients were able to walk further with less dyspnea; however, the standard deviation of both parameters was greater than the absolute differences from the baseline values to those at the conclusion of the study at 12 weeks, leading to difficulty in interpreting the efficacy of the treatment.[32]

Because patients with cardiopulmonary disorders are usually more symptomatic during exertion, the most direct approach to an evaluation of functional capacity is to inquire about symptoms at rest and during exertion. The majority of exercise tests are designed to evaluate exercise performance at maximal workloads, but daily activities do not generally require energy expenditure in the maximal range. The SAS that we used in the present study quantitatively expresses exercise capacity in terms of energy cost of physical activities and this scale has been shown to linearly correlate with peak oxygen consumption. The reproducibility of measurement was substantial with a mean difference of 0.4±0.5 METs in interobserver variability,[17] prompting us to consider changes greater than 1 MET as clinically relevant. In the present study, SAS increased continuously and significantly throughout the study period, the mean change of 1.7±1.4 indicating a significant treatment effect in favor of bosentan.

In the placebo-controlled studies reported in the literature, treatment with 125 mg of bosentan twice daily was not associated with significant adverse events when compared with placebo.[9,10] However, increased doses led to a frequent elevation of aminotransferase concentrations in accord with the known incidence of abnormal hepatic function.[10] In the present study, 3 patients had increases in aminotransferase with bosentan at a dose of 125 mg twice daily and another 4 patients and 1 patients had increases at doses of 62.5 mg twice daily and 62.5 mg once daily, respectively. In those cases, the abnormal hepatic function progressively improved during bosentan therapy continued at either the same dose or at a reduced dose, except for one case in whom drug withdrawal was necessary. Liver injury induced by bosentan and its metabolites is thought to be mediated through inhibition of the canalicular bile salt export pump (BSEP), as evidenced by a dose-dependent increase in serum bile salts and alkaline phosphatase concentrations in a significant percentage of bosentan-treated patients, the increased cholestatic potency of bosentan with concomitant administration of a known BSEP inhibitor, the reproduction of similar effects in the experimental setting, or in vitro observation of inhibition of BSEP-mediated taurocholate transport by bosentan and metabolites.[33] Recently, it has also been reported that individual differences in susceptibility to the development of intrahepatic cholestasis observed during pregnancy are related to genetic variability in the gene encoding the BSEP.[34] Therefore, if detection of the responsible BSEP and other transporter polymorphisms becomes possible in future, individual susceptibility to drug-induced hepatotoxicity may be predicted.

In conclusion, there are no ethnic differences in the pharmacokinetics of bosentan, and dose adjustment is not necessary for Japanese patients. Japanese patients with severe pulmonary hypertension showed a significant improvement in cardiopulmonary hemodynamics, symptoms, and functional capacity over a 12-week treatment regimen of bosentan 125 mg twice daily. Aminotransferase concentrations were elevated in some cases but mostly returned to normal without discontinuation of therapy. Therefore, bosentan 125 mg twice daily is considered the clinically

preferable dose and is a valuable treatment option for Japanese patients with pulmonary hypertension, though close monitoring of liver function is necessary.

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Appendix 1

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Altered intracellular Ca^{2+} handling in heart failure

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Structural and functional alterations in the Ca^{2+} regulatory proteins present in the sarcoplasmic reticulum have recently been shown to be strongly involved in the pathogenesis of heart failure. Chronic activation of the sympathetic nervous system or of the renin-angiotensin system induces abnormalities in both the function and structure of these proteins. We review here the considerable body of evidence that has accumulated to support the notion that such abnormalities contribute to a defectiveness of contractile performance and hence to the progression of heart failure.

Introduction

Heart failure (HF) is characterized by a complex disorder that leads to a disturbance of the normal pumping of blood to the peripheral organs to meet the metabolic demands of the body. In a heart that has suffered myocardial damage, regardless of the initial cause of the damage (hypertension, myocardial ischemia, cardiomyopathy, etc.), HF eventually occurs if such damage persists for a prolonged period (1, 2). In the initial stages, compensation for the myocardial damage and maintenance of hemodynamics can occur via activation of both the sympathetic nervous system and the renin-angiotensin system, resulting in LV dilatation and/or hypertrophy. However, if the depressed cardiac function persists, with a parallel activation of neurohumoral factors, the myocardial damage becomes progressive and irreversible, and the heart can no longer meet the metabolic demand of the body, resulting in the phenotype of HF (1, 2).

A growing body of evidence has accumulated concerning the altered intracellular Ca^{2+} cycling that plays a key role in the development of HF (3–5). Recent advances in the field of molecular biology have shed light on the close relationship between Ca^{2+} cycling abnormalities and the progression of HF. In many cases, altered Ca^{2+} cycling precedes the observed depression of mechanical performance; consequently, an amelioration of the disorder of Ca^{2+} cycling has potential as a new and intriguing therapeutic strategy against HF (5). In this review, we focus on the role of Ca^{2+} regulatory proteins in the pathogenesis of HF and on the possibility of developing a new therapeutic strategy against HF using Ca^{2+} regulatory proteins as the target.

Intracellular Ca^{2+} handling in normal cardiomyocytes

In the normal heart, intracellular Ca^{2+} movements critically regulate subsequent mechanical contractions. In cardiac excitation-contraction (E-C) coupling (Figure 1), a small amount of Ca^{2+} first enters through the L-type Ca^{2+} channel (LTCC) during membrane depolarization. This Ca^{2+} influx triggers a large-scale Ca^{2+} release through the Ca^{2+} release channel of the sarcoplasmic reticulum (SR), referred to as the ryanodine receptor (RyR). The released Ca^{2+} then binds to the troponin C within the myofilaments, which induces activation of the myofilaments and a consequent muscle contraction (6–8). Relaxation is initiated by dissociation of Ca^{2+} from troponin C, followed by its reuptake into the SR through phospholamban-regulated (PLN-regulated) Ca^{2+}-ATPase (SERCA2a) and subsequent trans-sarcolemmal Ca^{2+} removal through the Na^+/Ca^{2+} exchanger (NCX) operating in its forward mode (7–9). The whole process of Ca^{2+} movement is characterized by a transient increase in intracellular $[Ca^{2+}]$ from 100 nM to about 1 μM (8). For termination of Ca^{2+} release, RyR adaptation (10), RyR inactivation (11), and SR Ca^{2+} depletion may play important roles by acting in a synergistic manner.

Triggers for Ca^{2+} release and defective E-C coupling in HF

In most types of HF, the density of LTCC seems to be either unaltered or reduced (12). However, there is evidence that the function of LTCC may be altered in human HF. For instance, Shroder et al. (13) demonstrated increases in both the availability and open probability of LTCC isolated from failing human hearts, possibly due to a defect in dephosphorylation. Moreover, Chen et al. (14) recently reported that the density of LTCC was reduced in human HF although the current was maintained due to an increase in the phosphorylation level. The efficiency of the trigger (the size of the inward Ca^{2+} current) needed to cause Ca^{2+} release from the SR has been termed E-C coupling gain (15). In many cases of HF, the E-C coupling gain seems to be reduced by several factors: (a) a functional defect in LTCC (16–18); (b) an increase in the space between LTCC and RyR (19); (c) a decrease in SR Ca^{2+} (20–22); and/or (d) an abnormality in the channel-gating property of RyR (23–26). Not only the amount of Ca^{2+} released for a given Ca^{2+} release trigger but also the rate of Ca^{2+} release may be important for the contractility of the myofilaments. Since crossbridge cycling is considered to occur very rapidly from the beginning of the rising phase of the Ca^{2+} transient (27), a faster elevation of the cytosolic Ca^{2+} concentration might accelerate crossbridge attachment, resulting in faster and/or higher tension development. In contrast, the dyssynchronous Ca^{2+} release seen in HF might lead to a slower rate of rise in the Ca^{2+} transient (28, 29), probably leading to a dyssynchronous binding of Ca^{2+} to troponin C, and thereby to a slower velocity of contraction (30). However, since there is no direct evidence to support this notion, it remains to be determined whether the reduced velocity of contraction in HF really is caused by an altered gain or efficiency of E-C coupling.

In HF, the SR Ca^{2+} content is reportedly decreased (20–22) although the fraction of Ca^{2+} released to Ca^{2+} sequestered during Ca^{2+} uptake seems to be increased (25). Both an upregulation of NCX and a

Nonstandard abbreviations used: ARVD/C2, arrhythmogenic right ventricular cardiomyopathy type 2; CAMKII, calmodulin-dependent kinase II; CCD, central core disease; CPVT, catecholaminergic polymorphic ventricular tachycardia; DAD, delayed after-depolarization; E-C, excitation-contraction; FKBP, FK506 binding protein; HF, heart failure; I-1, inhibitor-1; LTCC, L-type Ca^{2+} channel; MH, malignant hyperthermia; MyBP-Cmut, myosin-binding protein C-mutant mouse; NCX, Na^+/Ca^{2+} exchanger; PKA, protein kinase A; PLN, phospholamban; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; RyR, ryanodine receptor; SERCA2a, Ca^{2+}-ATPase; SR, sarcoplasmic reticulum.

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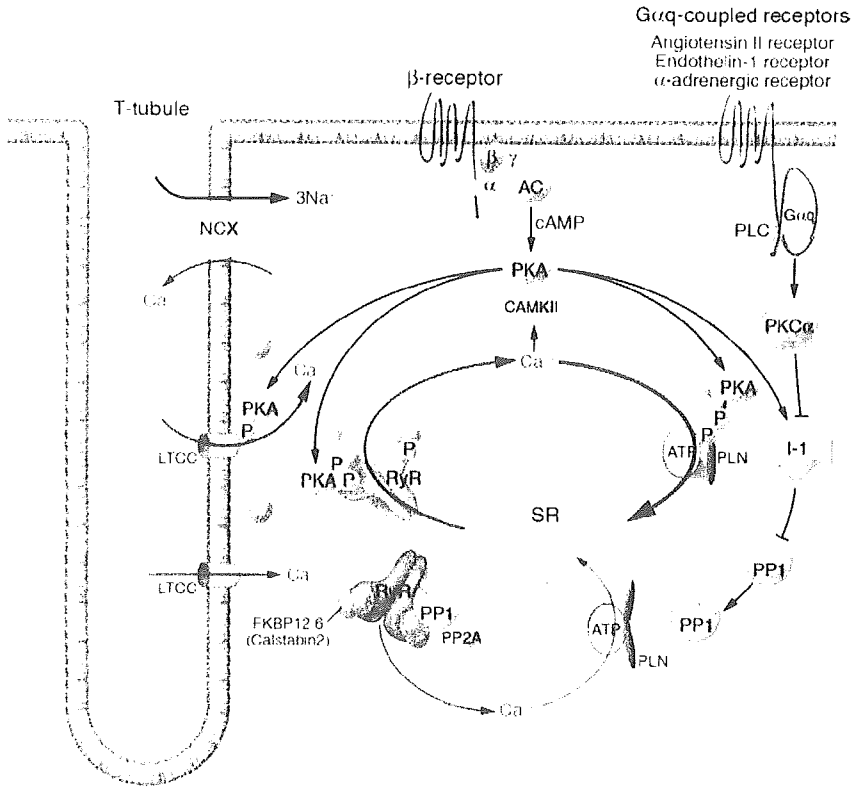

Figure 1

Intracellular Ca^{2+} cycling and associated signaling pathway in cardiomyocytes. On a beat-by-beat basis, a calcium transient is evoked by the initial influx of a small amount of Ca^{2+} through the LTCC and the subsequent large-scale Ca^{2+} release from the SR through the RyR. During diastole, cytosolic Ca^{2+} is taken up into the SR by the PLN-regulated SERCA2a pump. β receptor–mediated PKA stimulation regulates this Ca^{2+} cycling by phosphorylating LTCC, RyR, and PLN. In normal hearts, sympathetic stimulation activates β_1-adrenergic receptor, which in turn stimulates the production of cAMP by adenylyl cyclase and thereby activates PKA. PKA phosphorylates PLN and RyR, both of which contribute to an increased intracellular Ca^{2+} transient and enhanced cellular contractility (pink zone signal). PP1 and PP2A regulate the dephosphorylation process of these Ca^{2+} regulatory proteins (RyR, PLN, LTCC) (blue zone signaling). Activation of the Gαq-coupled receptors (angiotensin II receptor, endothelin 1 receptor, or α-adrenergic receptor) activates PLC, which in turn activates PKC-α. The PKC-α phosphorylates I-1, augmenting the activity of PP1 and causing hypophosphorylation of PLB. The PLB hypophosphorylation inhibits SERCA2a activity, thereby decreasing SR Ca^{2+} uptake. The increased Ca^{2+} level in the cytosol activates CAMKII, which affects the functions of RyR and PLN. Activation or deactivation of these molecules at a node in the signaling cascade affects beat-by-beat Ca^{2+} cycling, and such maneuvers have recently been highlighted as potential new therapeutic strategies against HF. α, G protein subunit α; β, G protein subunit β; γ, G protein subunit γ; AC, adenylyl cyclase; PLC, phospholipase C.

reduction in SERCA2a activity may be responsible for the reduced SR Ca^{2+} content observed in HF (12). The depressed SR Ca^{2+} load would reduce the E-C coupling gain, leading to contractile dysfunction as described above. In the normal contractile state, the greater SR Ca^{2+} content leads to a large fraction of the SR Ca^{2+} being released for a given Ca^{2+} trigger (7). This may be attributable to a stimulatory effect of the high intraluminal $[Ca^{2+}]$ ($[Ca^{2+}]$SR) on the channel open probability of RyRs (7, 31). Since the SR Ca^{2+} content is reduced in HF, the threshold SR content for a fractional Ca^{2+} release may be reduced, leading to a susceptibility to aberrant Ca^{2+} release (or spontaneous Ca^{2+} leak) at lower cytosolic $[Ca^{2+}]$. RyRs are coupled to proteins at the luminal SR surface (triadin, junctin, and calsequestrin) (32). Since these proteins buffer luminal Ca^{2+} and modulate the Ca^{2+} release process (32), structural and functional alterations in these proteins may be causally involved in the development of defective intraluminal $[Ca^{2+}]$ regulation seen in HF. A hypersensitivity of RyR2 channel opening to cytosolic $[Ca^{2+}]$ may contribute to the presence of a spontaneous Ca^{2+} leak at much lower levels of cytosolic $[Ca^{2+}]$ in HF than in the normal SR (i.e., approximately 100 nM during diastole). This spontaneous Ca^{2+} leak may lead to a delayed after-depolarization (DAD), which can trigger arrhythmia (33).

Altered function of SR Ca^{2+} regulatory proteins in HF

Defective FKBP12.6-mediated stabilization of RyR as a cause of HF. RyR is a Ca^{2+} release channel existing as a huge homotetramer transversing the SR membrane (34) (Figure 2). Three mammalian isoforms of RyR have been identified. Of these, RyR1 is found in skeletal muscle while RyR2 is predominantly expressed in cardiac muscle (35, 36). RyR3 is ubiquitously expressed at low levels and has functional properties that differ from those of both RyR1 and RyR2 (37).

Each monomer contains approximately 5,000 amino acids and has a molecular weight of 565 kDa. RyR is also a scaffolding protein to which numerous key regulatory proteins are bound, thus forming the junctional complex (38–40). It associates with FK506-binding protein (FKBP), calmodulin, protein kinase A (PKA), protein phosphatase 1 (PP1), and protein phosphatase 2A (PP2A). The RyRs are closely associated with LTCC, and this spatial association of the 2 channels forms a key functional unit in cardiac E-C coupling (32).

One of these accessory proteins, FKBP12.6, plays an important role in the stabilization of the channel (in other words, in the maintenance of the closed state of the channel). FKBP12.6 binds to RyR2 with a stoichiometric ratio of 1 FKBP12.6 to 1 RyR2 monomer, or 4 FKBP12.6s to 1 tetramer (41). Marx et al. (39) reported that in human HF, and in an experimental model of HF, PKA-mediated hyperphosphorylation of RyR2 occurs, and this in turn dissociates FKBP12.6 from RyR2, leading to a diastolic Ca^{2+} leak through RyR2. Long-term hyperphosphorylation of RyR2 can be maintained through a reduction in the protein abundance of PP1 and PP2A, both of which are tightly coupled to RyR2 (39). In a lipid-bilayer experiment, single-channel activity was found to be hypersensitized to $[Ca^{2+}]$, owing to a partial loss of FKBP12.6 from RyR2, thus causing (in HF) a diastolic Ca^{2+} leak at a concentration of $[Ca^{2+}]$ (approximately 100 nM) at which no significant Ca^{2+} release is induced in normal hearts (39). This diastolic Ca^{2+} leak depresses the SR Ca^{2+}

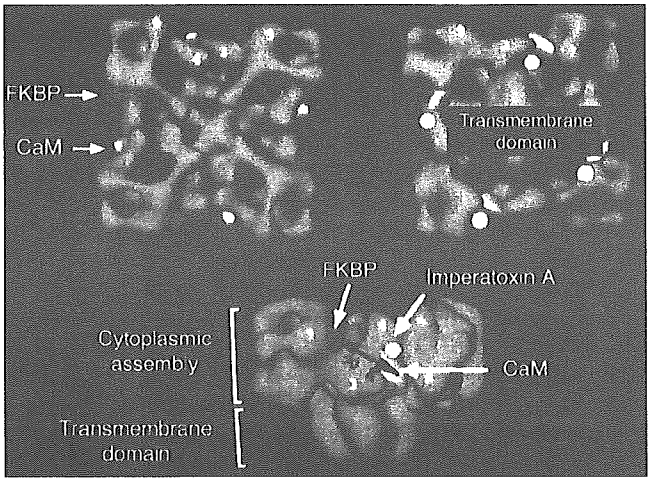

Figure 2
Three-dimensional structure of the skeletal muscle RyR, with some key sites of protein interactions. FKBP, FK506-binding protein; CaM, calmodulin. Image reprinted with permission from the *Journal of Biological Chemistry* (115).

load and serves as a substrate for DAD, which can trigger cardiac arrhythmia and lead to sudden death (42–44). The dissociation of FKBP12.6 from RyR2 also functionally uncouples multiple RyR2s and disturbs both the simultaneous opening of RyR2s during systole and their simultaneous closing during diastole (26, 39). In vivo, Shannon et al. (45) did indeed find a diastolic Ca^{2+} leak in a rabbit model of myocardial infarction. Earlier, we found that in a canine model of pacing-induced HF, PKA-hyperphosphorylation of RyR2 occurred in association with a conformational change in RyR2 and a subsequent prominent Ca^{2+} leak through RyR2 (23), although Jiang et al. later obtained conflicting results using the same model (46).

Attempts to reproduce the altered channel-gating property seen in HF have not been successful in intact myocytes. Independent groups have reported that phosphorylation at serine2808 or serine2809 does not cause FKBP12.6 dissociation from RyR2 and that the constitutive phosphorylation of serine2808 or serine2809 by mutations (S2808D or S2809D) fails to disrupt the FKBP12.6-RyR2 interaction (47, 48). To explain these apparently contradictory findings, Wehrens et al. (49) recently provided data suggesting that overexpression of FKBP12.6 outside the physiological range (47, 48) overwhelms the shift in FKBP12.6-binding affinity induced by PKA phosphorylation, allowing FKBP12.6 to bind to PKA-phosphorylated RyR2. Regarding other findings that seem to conflict with the PKA-hyperphosphorylation theory of HF (39), Li et al. (50) found that PKA phosphorylation of RyR did not increase calcium sparks in permeabilized myocytes. However, this study was performed under conditions in which cytosolic Ca^{2+} is clamped at 50 or 10 nM, which is lower than diastolic Ca^{2+} concentrations. It appears that this may match the physiology of E-C coupling because increased Ca^{2+} release under resting diastolic conditions would cause a serious problem. Valdivia et al. (51) showed that PKA phosphorylation caused an initial transient RyR2 opening in response to a jump in $[Ca^{2+}]$, followed by a rapid deactivation of channel gating. Eisner et al. (52) showed that the abrupt increase in RyR2 opening induced by caffeine in intact cells has only transient effects on the amplitude of the Ca^{2+} transient (due to autoregulation). That is to say, the additional Ca^{2+} released by enhanced RyR2 opening will be rapidly removed by NCX during the subsequent beat, thereby reducing the SR Ca^{2+} available for the next beat. In the steady state, the reduced SR Ca^{2+} content offsets the effects of increased RyR2 opening with the result that Ca^{2+} transients are almost unchanged. These studies may provide a mechanism for transiently increasing systolic SR Ca^{2+} release in a physiological manner to increase cardiac contractility. An important problem in HF is that RyR2s are chronically PKA hyperphosphorylated with a partial loss of FKBP12.6 and that as a result the channels become leaky. These leaky RyR2 channels may reduce the SR Ca^{2+} load and in turn lead to the reduced contractility of cardiac muscle in failing hearts.

Mutations within RyR as a cause of defective channel opening. More than 40 RyR1 mutations have been found in patients with malignant hyperthermia (MH) or central core disease (CCD) (33). It has been shown that such RyR1 mutations in MH and CCD produce an abnormal mode of channel gating that alters the Ca^{2+} inactivation process and makes the channel hyper- and hyposensitive to activating and inactivating ligands, respectively (53). The mutation sites cluster into 3 major regions (N-terminal, central, and C-terminal). To date, more than 30 mutations have been found in the analogous RyR2 regions in patients with arrhythmogenic right ventricular cardiomyopathy type 2 (ARVD/C2) or catecholaminergic polymorphic ventricular tachycardia (CPVT) (54–63) (Table 1). This suggests that these 3 regions represent domains that are critical for the regulation of both RyR1 and RyR2 and that these domains are also involved in the pathogenesis of RyR-linked skeletal and cardiac muscle diseases. Interestingly, 1 of the cardiomyopathy (ARVD/C2) mutations in the N-terminal domain of RyR2, Arg176Gln, corresponds exactly to the Arg163Cys human MH mutation of RyR1. Likewise, some RyR2 mutations either match exactly or are located very close to some of the mutations found in RyR1.

The above findings strongly suggest that each mutation site is crucial for the maintenance of the normal channel-gating property. Marks and colleagues recently investigated the pathogenic role of RyR2 mutations by evaluating channel activity in recombinant RyR2 containing the same single-point mutation as that seen in CPVT patients (64). They found that FKBP12.6-deficient mice and CPVT-associated RyR2 mutants exhibited a significantly increased open probability of the channel only during exercise or in the PKA-phosphorylated state, that these RyR2 mutants displayed a reduced affinity of FKBP12.6 for RyR2, and that a constitutively active recombinant FKBP12.6 (FKBP12.6-D37S, a mutant form of FKBP12.6 with serine residue 37 substituted for aspartic acid) that can bind to PKA-phosphorylated RyR2 reversed the hyperactivity of channel gating seen in PKA-phosphorylated mutated RyR2 (64). These finding are compatible with the clinical finding that CPVT patients do not exhibit arrhythmia at rest but may suffer lethal arrhythmia during exercise (65).

Recently, Jiang et al. (66) reported that the mutant RyR2 linked to CPVT and sudden death increased the sensitivity of single RyR2 channels to activation by luminal Ca^{2+} and enhanced the basal level of [^{3}H]ryanodine binding, even without PKA-phosphorylation. The discrepancies between these reports might be partly explained by FKBP12.6 being absent from the RyR2 mutant studied by Jiang et al. (66) but present in the RyR2 mutant studied by Wehrens et al. (64). It remains to be determined whether the resting channel-gating property of the FKBP12.6-depleted, mutant RyR2 linked to

Table 1
RyR2 mutations found in patients with arrhythmogenic right ventricular cardiomyopathy and CPVT

Amino acid	Disease	Domain	References
R176Q	ARVD/C2	N-terminal	(56)
R414L	CPVT	N-terminal	(62)
I419F	CPVT	N-terminal	(62)
R420W	ARVD/C2	N-terminal	(61, 57)
L433P	ARVD/C2	N-terminal	(56)
S2246L	CPVT	Central	(54, 59, 61)
V2306I	CPVT	Central	(58)
R2311D	CPVT	Central	(59)
P2328S	CPVT	Central	(55)
N2386I	ARVD/C2	Central	(56)
A2387P	CPVT	Central	(60)
Y2392C	ARVD/C2	Central	(57)
A2403T	CPVT	Central	(62)
R2474S	CPVT	Central	(54)
T2504M	ARVD/C2	Central	(56)
L2534V	CPVT	Central	(63)
L3778F	CPVT	C-terminal	(59)
G3946S	CPVT	C-terminal	(59)
N4097S	CPVT	C-terminal	(61)
N4104K	CPVT	C-terminal	(54)
E4146K	CPVT	C-terminal	(61)
T4158P	CPVT	C-terminal	(61)
Q4201R	CPVT	C-terminal	(55)
R4497C	CPVT	C-terminal	(54, 61)
F4499C	CPVT	C-terminal	(62)
N4504I	CPVT	C-terminal	(60)
A4510T	CPVT	C-terminal	(62)
A4608P	CPVT	C-terminal	(60)
V4653F	CPVT	C-terminal	(55)
G4671R	CPVT	C-terminal	(62)
V4771I	CPVT	C-terminal	(59)
I4848V	CPVT	C-terminal	(62)
A4860G	CPVT	C-terminal	(59)
I4867M	CPVT	C-terminal	(59)
V4880A	CPVT	C-terminal	(60)
N4895D	CPVT	C-terminal	(59)
P4902L	CPVT	C-terminal	(58)
E4950K	CPVT	C-terminal	(59)
R4959Q	CPVT	C-terminal	(58)

The reported mutations cluster in 3 regions homologous to 3 MH/CCD regions (that is, N-terminal, central, and C-terminal regions).

CPVT and sudden cardiac death can be altered even without PKA phosphorylation and how PKA phosphorylation affects the channel activity in the FKBP12.6-depleted, mutant RyR2.

Domain-domain interaction: a key mechanism for stabilization of RyR. On the grounds that mutations in either the N-terminal or the central domain produce abnormal modes of RyR channel gating, generally referred to as hyperactivation and hypersensitization effects (33, 53), Ikemoto et al. (67) proposed an intriguing hypothesis. In this hypothesis, the 2 domains (N-terminal and central domain) interact with each other to act as a regulatory switch for channel-gating activity, with a tight zipping of the interacting domains serving to stabilize the channel. A mutation in either domain weakens the interdomain interaction, thus increasing the tendency toward unzipping, which causes activation and leakiness of the Ca^{2+} channel (67). For instance, 1 of the domain peptides, DP4, which corresponds to the Leu2442-Pro2477 region of the central domain, has been found to enhance [^{3}H]ryanodine binding and to induce Ca^{2+} release from the SR, thereby inducing contraction, in skinned muscle fibers at an inhibitory Mg^{2+} concentration (68). DP4 is also known to increase the frequency of Ca^{2+} sparks in saponin-permeabilized fibers (69) and to increase the open probability of single channels (69). In addition, a cardiac domain peptide corresponding to the Gly2459-Pro2494 region of RyR2 (DPc10) has been shown to produce significant activation of the RyR2 Ca^{2+} channel at low Ca^{2+} concentrations in a way similar to that described for DP4 (70). An Arg-to-Ser mutation in the peptide that mimics the Arg2474-to-Ser2474 human CPVT mutation completely abolished both the hyperactivation and the hypersensitization effects seen with DPc10 (70). In light of these data, it might be anticipated that a mutation in the Gly2460-Pro2495 domain of RyR2 would not only make the Ca^{2+} channel leaky but also increase its sensitivity to various pharmacological agonists, leading to diastolic Ca^{2+} overload, as widely seen in failing hearts. Since the binding region of FKBP12.6 to RyR2, which seems to reside in residues 2361–2496 according to Marx et al. (39), is included in this sequence in DPc10 (residues 2460–2495), there may be a close mechanistic relationship between PKA-mediated FKBP12.6 dissociation and domain-domain interaction.

NCX. In intact hearts, trans-sarcolemmal Ca^{2+} removal occurs through the NCX, acting in its forward mode (8). In hypertrophied or failing hearts, cytosolic [Na^+] has been shown to be elevated (71–73), leading to activation of the reverse mode of NCX, which causes a Ca^{2+} influx (74). Although this Ca^{2+} influx via NCX is not as efficient for triggering Ca^{2+} release as the inward Ca^{2+} current through LTCC (75), it may contribute to an increase in SR Ca^{2+} content that is favorable for an increase in contractility. In failing hearts, the increased Ca^{2+} influx via the reverse mode of NCX may not be completely taken up during diastole by SERCA2a, owing to its decreased activity, resulting in the increase in diastolic [Ca^{2+}] that leads to impairment of relaxation (74). As heart rate increases through an activation in sympathetic tone, cytosolic [Na^+] is elevated, and the subsequent rise in Ca^{2+} influx via NCX contributes to a frequency-dependent increase in contractility (positive staircase) (72). In failing hearts, this response is blunted, and the diastolic [Ca^{2+}] is elevated due to the combined effect of a decreased SR Ca^{2+} uptake and an additional Ca^{2+} influx via NCX in its reverse mode (74). Moreover, the increased Ca^{2+} influx that occurs during the later phase of the action potential causes a tail Ca^{2+} transient that induces DAD and triggers arrhythmia (76). The abundance of NCX protein is reportedly increased in both experimental and human failing hearts although some studies showed an unchanged level (12). This may possibly be explained by differences in the stage and/or severity of the HF.

SERCA2a and PLN. Many studies have demonstrated a reduced expression of SERCA2a protein in failing hearts, although some studies have shown an unaltered expression (12). Consistently, previous studies have indicated that SR Ca^{2+} uptake (or SERCA2a activity) is reduced in the failing animal or human myocardium (25, 72, 77). A decrease in the PLN mRNA level has been consistently observed in failing hearts. However, some studies report that the PLN protein level was unchanged (12), resulting in the protein expression of SERCA2a relative to PLN being diminished (3, 4). This ratio (i.e., the protein expression of SERCA2a relative to PLN) indicates the extent of Ca^{2+} pump inhibition, and hence the basal level of SERCA2a activity is at a lower level in failing hearts than in normal hearts (3, 4). Regarding the phosphorylation

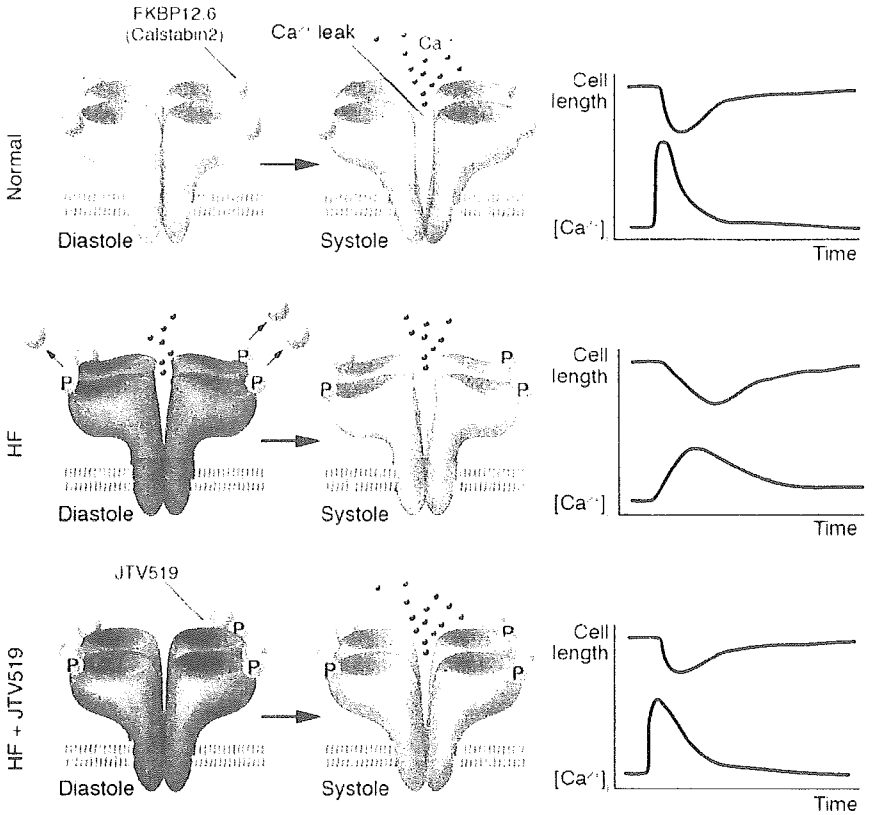

Figure 3
Therapeutic strategy involving FKBP12.6-mediated stabilization of RyR. A small influx of Ca^{2+} through the LTCC leads to the release of a large amount of Ca^{2+} from the SR through RyR in the normal heart. In HF, however, PKA-mediated hyperphosphorylation of RyR2 occurs, and this in turn dissociates FKBP12.6 from RyR2, leading to a diastolic Ca^{2+} leak through RyR2. This results in the Ca^{2+} transient being diminished (due to the reduced SR Ca^{2+} content and dyssynchronous Ca^{2+} release). Administration of a new compound, the 1,4-benzothiazepine derivative JTV519, normalizes this abnormal channel gating by restoring the conformational state of RyR and by rebinding FKBP12.6 to the channel complex. Thereby, JTV519 normalizes Ca^{2+} cycling and contractile function in failing cardiac myocytes and hence provides chronic suppression of progressive left ventricular dysfunction in HF. P, PKA phosphorylation at serine 2809; $[Ca^{2+}]_i$, intracellular $[Ca^{2+}]$.

of PLN, the level of serine 16 phosphorylation has variously been reported to be reduced (78–80) or unaltered (81, 82) in HF whereas the level of threonine 17 phosphorylation has consistently been reported to be decreased (82, 83). Threonine 17 phosphorylation is affected by the decreased calmodulin-dependent kinase II (CAMKII) activity in HF whereas serine 16 phosphorylation is mainly affected by PKA activity. The level of CAMKII activity also affects the ser-38 residue, which is the calcium-binding domain of SERCA2a, thereby regulating calcium uptake (84). The altered phosphorylation state of PLN may be responsible for the reduced SR calcium-uptake activity seen in HF. Type-1 PP1, which makes up a major protein of the serine/threonine protein phosphatases present in the cardiac myocyte, may also play an important role in regulating PLN phosphorylation since PP1 has been shown to be hyperactivated concurrently with a reduced level of serine 16 phosphorylation in PLN in several models of HF (85–87) and since overexpression of PP1 catalytic subunit α in the mouse heart was shown to lead to marked left ventricular dilation and premature death due to severe HF (88). Decreased threonine 35 phosphorylation in inhibitor-1 (I-1), an endogenous inhibitor of PP1, is further associated with increased PP1 activity in the failing heart (88).

A recent report by Braz et al. (89) demonstrated that PKC regulation of PP1 activity is critical. In HF, not only is the β-adrenergic system stimulated, but the receptor-operated signalings triggered by angiotensin II, endothelin, and the α-adrenergic system are chronically activated, contributing to depressed contractility and to a progression of both remodeling and apoptosis (1, 2). The common key enzyme in the downstream events in these receptor-operated systems is PKC. It has been demonstrated that PKC-α, which is the predominant PKC isoenzyme expressed in the heart (90), plays a key role in regulating cardiac contractility and Ca^{2+} handling in myocytes (89). PKC-α directly phosphorylates serine 67 in I-1, thereby augmenting the activity of PP1 and causing hypophosphorylation of PLN (89). This finding may in part answer the question of why SERCA2a activity is found to be reduced in HF, apart from the more obvious possibility of a reduced abundance of SERCA2a protein.

New treatment for HF by modulation of Ca^{2+} regulatory proteins

Stabilization of RyR. The RyR2 has been shown to be hyperphosphorylated by PKA in both human and experimental HF (23, 39, 91–94), although admittedly Jiang et al. did not observe PKA hyperphosphorylation of RyR2 in a canine model of HF (46). Many large clinical trials have shown that treatment with a β blocker restores cardiac function and reduces the rate of mortality in patients with HF (2, 95). Several researchers have reported recently that in experimental and human HF, β blockers reversed PKA-mediated hyperphosphorylation of RyR2, restored the stoichiometry of the RyR2 macromolecular complex, restored normal single-channel function, and inhibited the Ca^{2+} leak (91–93). These findings may provide a molecular basis for the common clinical observation that the use of β receptor blockers improves the prognosis of patients with HF. In a canine model of HF, we found that the angiotensin II receptor blocker valsartan, which has been used in the treatment of HF in the clinical setting, also normalizes the Ca^{2+} regulatory process through a β blocker–like action (94). By acting on the presynaptic angiotensin II receptor, valsartan inhibited norepinephrine release and stimulated norepinephrine uptake back into the synaptic pool, with the result that adrenergic signals were

Table 2
Effect of in vivo molecular intervention upregulating SR Ca^{2+} uptake in animal models of HF

Model	Evaluated phenotype	Target gene	Rescue effect	References
SERCA2a				
Aortic-banded rat	HF	SERCA2a GT	Yes	(97)
Aortic-banded rat	Metabolism and survival	SERCA2a GT	Yes	(98)
Aortic-banded mouse	LV dysfunction	SERCA2a Tg	Yes	(100)
Diabetic cardiomyopathy rat	LV dysfunction	SERCA2a Tg	Yes	(99)
Aortic-banded mouse	LV hypertrophy and HF	SERCA2a Tg	Yes	(101)
Aortic-banded rat	HF	SERCA2a Tg	Yes	(102)
Aortic-banded rat	Arrhythmia	SERCA2a GT	Yes	(103)
PLN				
MLPKO mouse	DCM and HF	PLN KO	Yes	(106)
Tropomodulin mutant Tg mouse	DCM and HF	PLN KO	No	(111)
Calsequestrin Tg mouse	LV hypertrophy and HF	PLN KO	Yes	(107)
MHC (R403Q) mutant Tg mouse	LV hypertrophy and HF	PLN KO	Yes	(108)
BIO14.6 hamster	DCM and HF	PLNS16E GT	Yes	(109)
Gaq Tg mouse	LV hypertrophy and HF	PLN KO	No	(112)
MyBP-C(Mut) Tg mouse	HCM and HF	PLN KO	No	(112)
Postinfarction rat	LV remodeling and HF	PLNS16E GT	Yes	(110)
PP1/PKC-α				
MLPKO mouse	DCM and HF	PKC-α KO	Yes	(89)

GT, gene transfer; HCM, hypertrophic cardiomyopathy; DCM, dilated cardiomyopathy.

not overtransmitted into the cell. This would lead to a reduction in the PKA-hyperphosphorylation of RyR2 and to an inhibition of the Ca^{2+} leak in the failing heart (94).

Since a conformational change in RyR2 precedes the Ca^{2+} leak (23), an amelioration of this conformational change could be a new therapeutic strategy against HF (Figure 3). Using a canine model of HF, we recently found that chronic administration of a new compound, the 1,4-benzothiazepine derivative JTV519, improved contractility and prevented the development of LV remodeling and HF, presumably by stabilization of RyR2 (80). In JTV519-untreated hearts, RyR2 was PKA-hyperphosphorylated with a dissociation of FKBP12.6 whereas the reverse of these states was true of JTV519-treated hearts, in which channel phosphorylation returned toward the levels seen in the normal heart (80). Using FKBP12.6$^{-/-}$ mice, Wehrens et al. (49) demonstrated that JTV519 increased the affinity of FKBP12.6 for RyR2, which stabilized the closed state of RyR2 and prevented the Ca^{2+} leak that triggers arrhythmias. In their study, FKBP12.6$^{-/-}$ mice showed an increase in RyR2 open probability, ventricular tachycardia, and sudden cardiac death upon either exercise or PKA-phosphorylation. JTV519 did not prevent arrhythmias in FKBP12.6$^{-/-}$ mice, indicating that the presence of FKBP12.6 in the heart is required for the therapeutic effects of JTV519 to be expressed (49), although it needs to be determined whether the same is true in FKBP12.6-depleted (by PKA-phosphorylation or FK506) RyR2. Lehnart et al. (96) found that recombinant RyR2 channels containing the missense mutations seen in CPVT patients (RyR2-P2328S, RyR2-Q4201R, and RyR2-V4653F) showed defective channel-gating properties (that is, an increase in open probability and resistance to Mg^{2+}-induced inhibition after PKA phosphorylation) and that JTV519 normalized this abnormal channel gating via a rebinding of FKBP12.6 to the channel complex. Collectively, the above data suggest that stabilization of RyR2 may represent a new molecular target for the treatment or prevention of exercise-induced arrhythmias and sudden death in patients with CPVT mutations and HF.

Overexpression of SERCA2a and PLN inhibition. One intriguing therapeutic approach to the treatment of HF may be to restore the depressed Ca^{2+} uptake activity that is generally considered to play a key pathogenic role in the development of this condition. The enhanced contractility associated with SERCA2a overexpression has been reported to be protective against both HF and cardiac hypertrophy (97–102). For example, in a pressure-overload rat model of HF, adenovirus-mediated overexpression of SERCA2a has been found to rescue depressed contractility and survival without adverse effects on energy metabolism (97, 98) and cardiac arrhythmia (103). The enhanced contractility induced by SERCA2a overexpression is due to an enhanced SR Ca^{2+} content and the resulting increase in Ca^{2+} efflux during systole (104, 105). An inhibition of PLN and the subsequent increase in SERCA2a activity appear to be promising strategies for the treatment of HF. Indeed, PLN gene ablation has been shown to prevent ventricular dysfunction, fibrosis, and development of HF both in dilated cardiomyopathy and hypertrophic cardiomyopathy (106–108). An in vivo gene transfer of a dominant-negative PLN mutant (which leads to greater SERCA2a activity) rescued HF both in a cardiomyopathic hamster model (109) and in rats with myocardial infarction (110). In contrast to these studies showing the functional benefit of enhanced SERCA2a activity in failing myocardium, other studies showed that PLN ablation failed to rescue the cardiomyopathic phenotype in several models of cardiomyopathy (i.e., tropomodulin-overexpressing transgenic [TOT] mouse [ref. 111], Gaq-transgenic mouse, and myosin-binding protein C mutant mouse [MyBP-Cmut] [ref. 112]). These data suggest that the ablation or inhibition of PLN may not always be effective for the treatment of HF (Table 2). Independent groups have reported that human PLN mutations lead to dilated cardiomyopathy with Arg9Cys (113) and Leu39stop mutations (114). The PLN with Arg9Cys mutation was

found to interact abnormally with PKA and lack the ability to be phosphorylated at Ser16, thereby dominantly inhibiting SERCA2a function (113). These data support the notion that PLN inhibition is a promising therapeutic approach for human HF. On the other hand, the fact that PLN with Leu39stop mutation lacks transmembrane peptide and thereby disables PLN retention in the SR (114) raises the possibility that innate absence of PLN in the SR may cause long-term adverse effects in the human heart. However, the lod score for the linkage between Leu39stop mutation and HF was low, and moreover, the physiological significance of Leu39stop mutation was not evaluated in the animal model. Further investigation is still needed to determine whether either overexpression of SERCA2a or PLN inhibition can be a new therapeutic strategy against human HF. It is also of great interest that, as recently demonstrated, overexpression of I-1 (88) or ablation of PKC-α (89) leads to increased myocyte contractility in the human failing myocyte and muscle LIM protein (MLP)–deficient cardiomyopathy, respectively, presumably by inhibiting the increased PP1 activity seen in the failing heart. Further assessment will be needed to determine whether PP1 inhibition might be beneficial in the long-term setting of HF.

Conclusions and perspectives

Recent progress in molecular cardiology makes it possible to envision a new therapeutic approach to HF, targeting key molecules involved in intracellular Ca^{2+} handling (such as RyR, SERCA2a, PLN, and others). Controlling these molecular functions has been found to be beneficial in certain experimental conditions. However, not all investigators are agreed that such therapies can usefully be extended to all types of failing hearts. Depending on the experimental conditions or on the model of HF, both positive and negative results (i.e., benefit or no benefit with the above therapies) have been obtained. With regard to this, it is important to notice whether the animal models accurately reflect the human disorder or the underlying human biology. Because of the heterogeneous nature of human HF examined in various studies (i.e., different stages and etiologies), caution should be exercised when trying to decide whether this approach might be generally applicable to the treatment of HF. In this regard, further investigation is clearly needed. Moreover, in contrast to many experimental situations, in which treatments are administered before HF develops, human HF has to be cured after it has developed. At present, little information is available to indicate whether manipulations targeting Ca^{2+} regulatory proteins are effective after HF has developed as well as before. Nevertheless, new forms of therapy targeting Ca^{2+} regulatory proteins should open up a new chapter in the potential treatment of HF.

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Original Article

The Combination Therapy of Hypertension to Prevent Cardiovascular Events (COPE) Trial: Rationale and Design

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A number of major clinical trials have demonstrated the clinical benefits of lowering blood pressure and have indicated that a majority of patients with hypertension will require more than one drug to achieve optimal blood pressure control. However, there is little data showing which antihypertensive combination best protects patients from cardiovascular events and which best achieves the target blood pressure with the fewest adverse events. The Combination Therapy of Hypertension to Prevent Cardiovascular Events (COPE) trial is the first large-scale investigator-initiated multicenter study with a prospective, randomized, open, blinded endpoint evaluation (PROBE) design to directly compare cardiovascular mortality and morbidity, incidence of adverse drug reaction, and degree of blood pressure reduction in Japanese hypertensive patients for a combination of angiotensin receptor blockers, β-blockers or thiazide diuretics in addition to a calcium antagonist, benidipine hydrochloride, with a response-dependent dose titration scheme. The COPE trial is being conducted with the cooperation of more than 100 centers and clinics in Japan and involves 3,000 patients, who will be followed for 3 years. Eligible patients are being enrolled from May 2003 until May 2006. Results from the COPE trial should provide new evidence for selecting optimal combination therapies for hypertensive patients. (*Hypertens Res* 2005; 28: 331–338)

Key Words: **hypertension, multicenter clinical trial, PROBE (prospective, randomized, open, blinded endpoint evaluation), combination therapy, benidipine**

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Introduction

Hypertension is an important risk factor for cardiovascular diseases (*1, 2*). The treatment goal for hypertension is decreasing morbidity and mortality by reducing organ damage and preventing cardiovascular complications. Large-scale clinical trials comparing various antihypertensives have been conducted to investigate their benefits in preventing cardiovascular events due to hypertension. They have shown that treatment with any commonly used regimen reduces the risk of major cardiovascular events and that increased reductions in blood pressure produce a better outcome in decreasing cardiovascular events and mortality (*3–5*). It remains uncertain whether the results obtained in Western countries can be directly applied to the Japanese population because of the differences in intrinsic/extrinsic racial factors between Western countries and Japan, such as the dosage of the drugs, the dietary habits, including higher salt intake (*6*), and the cardiovascular event rates (*7, 8*). In addition, various combinations of antihypertensive agents are often required to achieve optimal blood pressure control (*9*). Recent clinical guidelines for the treatment of hypertension recommend the combination therapy to achieve optimal blood pressure control (*3, 10–12*). However, the effects of the combination of antihypertensive drugs have not been well investigated in regard to morbidity and mortality in patients with hypertension. Therefore, it is important to determine which combinations will achieve an optimal outcome with the fewest side effects.

Rationale

It has been reported that all antihypertensive drugs have similar long-term efficacy and safety for the prevention of cardiovascular events in patients with hypertension (*4*), with calcium antagonists being especially effective in stroke prevention (*4, 13–15*). The Japanese Society of Hypertension Guidelines for Management of Hypertension in 2000 (JSH 2000) recommends calcium antagonists as a first-line drug for the treatment of hypertension (*12*). Prescription rates of the antihypertensive drugs in Japan are reported as follows: calcium antagonists, 73.0%; angiotensin-converting enzyme (ACE) inhibitors, 31.3%; angiotensin receptor blockers, 18.9%; β-blockers, 16.2%; and diuretics, 10.1% (*16*). Recent surveys in Japan revealed that only 50% of the subjects under antihypertensive treatment achieved the targeted blood pressure levels (*17, 18*). Although selection of some antihypertensive drugs was based on evidence from previous trials on hypertensive patients with diabetes mellitus, chronic heart failure, and renal insufficiency, calcium antagonists were selected for most age groups and in most patients with various complications associated with hypertension. Japanese doctors do not appear to consider age or complications when choosing antihypertensive regimens (*16*). Meta-analysis of 354 randomized double-blind placebo-controlled trials (*19*) demonstrated that the blood pressure reduction effects of different categories of drugs were additive, and symptoms attributable to thiazides, β-blockers, and calcium antagonists were strongly dose-related, whereas angiotensin receptor blockers caused no increase in symptoms. Furthermore, the prevalence of symptoms with two drugs in combination was less than additive, and adverse metabolic effects (such as changes in cholesterol or potassium) were negligible at a half-standard dose, indicating that low-dose combination treatment increases efficacy and reduces adverse drug reactions (*19*). In Japan, Saito *et al.* reported that the most common first-choice drugs by hypertension specialists were a calcium antagonist (69%) or an ACE inhibitor (22%). They further described that 72% selected a calcium antagonist and an ACE inhibitor as a combination therapy, and 17% selected a calcium antagonist and angiotensin receptor blocker as their first-choice drug combination (*20*). Since calcium antagonists are widely and successfully used for the treatment of hypertension in Japan (*21*), it is most likely that combination therapy with a calcium antagonist and some other antihypertensive drug will be chosen as the first-choice combination therapy for the treatment of hypertension. In addition, in Japan, as opposed to Western countries, cerebrovascular events, which are significantly positively related to hypertension, are more frequent than cardiovascular events (*7*). According to the current status of hypertension treatment in Japan, as stated above, the Combination Therapy of Hypertension to Prevent Cardiovascular Events (COPE) trial was designed to investigate, in patients with hypertension, which combination of antihypertensive drugs—angiotensin receptor blockers, β-blockers, or thiazide diuretics in addition to a long-acting calcium antagonist, benidipine hydrochloride—is superior for achieving the targeted blood pressure and preventing cardiovascular events with the fewest adverse drug effects. The COPE trial is conducted as a collaborative research project between Yamaguchi University and Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan).

Table 1. Inclusion Criteria for the COPE Trial

1. Outpatients who are required a combination therapy with sitting systolic blood pressure ≥140 mmHg or diastolic blood pressure ≥90 mmHg
2. Outpatients aged over 40 years and less than 85 years (inclusive), regardless of sex
3. Previously untreated patients or patients who are on other therapy, which can be converted to 4 mg of benidipine
4. Patients who can be treated with benidipine, angiotensin receptor blockers, β-blockers, and thiazide diuretics

COPE, Combination Therapy of Hypertension to Prevent Cardiovascular Events.

Ethics Committee Procedure and Consent

The study protocol was approved by the ethics committees of

Table 2. Exclusion Criteria for the COPE Trial

1.	Seated systolic blood pressure ≥200 mmHg or seated diastolic blood pressure ≥120 mmHg
2.	Secondary hypertension
3.	Type 1 diabetes mellitus or type 2 diabetes on insulin treatment
4.	History of cerebrovascular disorder, myocardial infarction, angina pectoris, coronary angioplasty or coronary artery bypass graft surgery within 6 months prior to enrolment in the study
5.	Heart failure (NYHA functional classification II, III or IV)
6.	Chronic atrial fibrillation or atrial flutter
7.	Congenital heart disease or a history of rheumatic heart disease
8.	Severe peripheral arterial disease (Fontaine Class II, III or IV)
9.	Serious liver dysfunction (AST or ALT ≥100 IU/l)
10.	Serious renal dysfunction (serum creatinine ≥2 mg/dl)
11.	History of malignancy 5 years prior to study entry
12.	Pregnancy
13.	Compliance rate <70% assessed by a patient interview
14.	Known hypersensitivity or contraindication to benidipine, angiotensin receptor blockers, β-blockers, and thiazide diuretics
15.	Other serious illness or significant abnormalities that the investigator judges inappropriate for the study

COPE, Combination Therapy of Hypertension to Prevent Cardiovascular Events; NYHA, New York Heart Association; AST, alanin aminotransferase; ALT, aspartate aminotransferase.

all institutions involved, and the trial was undertaken in accordance with the Declaration of Helsinki and the Ethical Guidelines for Clinical Studies in Japan (2003, Ministry of Health Labour Welfare of Japan: http://www.imcj.go.jp/rinri/index.html). All patients gave fully informed written consent.

Study Design

The COPE trial is an investigator-initiated, multi-center study with a prospective, randomized, open, blinded endpoint evaluation (PROBE) design (*22*) with a response-dependent dose titration scheme in Japanese hypertensive patients, who are prescribed a long-acting dihydropyridine calcium antagonist, benidipine, as an initial drug, and then assigned to receive either an angiotensin receptor blocker, β-blocker, or thiazide diuretic as a combination therapy. Benidipine has been widely used and proved beneficial for the treatment of hypertension (*23–25*). The target blood pressure of the COPE trial is less than 140/90 mmHg.

Patient Recruitment

Enrollment of eligible patients began in May 2003 and will continue through May 2006, and follow-up will be continued until May 2009. Inclusion and exclusion criteria are shown in Tables 1 and 2. The design outline of the COPE trial is shown in Fig. 1. Patients already receiving antihypertensive treatment are directly enrolled in the run-in phase of the COPE trial, discontinuing previous drugs and starting at an initial dosage of 4 mg of benidipine (step 0). All patients are required to be treated with benidipine for at least 4 consecutive weeks during the screening and run-in phases. During the run-in phase, blood pressure levels and compliance with benidipine are monitored. If the average blood pressure level at the last baseline visit is equal to or more than 140/90 mmHg and compliance is 70% or more, patients will be randomly assigned to receive an angiotensin receptor blocker, β-blocker, or thiazide diuretic and the initial dosage of benidipine as in step 1. Eligible patients are randomly assigned to one of the three study arms. The randomization is conducted at the COPE Trial Data Center by the dynamic allocation method (the modified minimization method) after stratification by regional block, health center, gender, age, systolic blood pressure (SBP), presence/absence of diabetes, and previous vascular events as adjusting factors in the minimization calculation.

Patient Follow-Up

Following randomization, patients receive follow-up visits over the remaining study period, during which safety parameters are checked annually, any adverse events are noted, and any interim endpoints are recorded. If a blood pressure target of <140/90 mmHg is not obtained, dose-titration is encouraged as shown in Fig. 1. A resting ECG is recorded and carotid bruit is examined at study entry and annually thereafter. During the course of the trial, if for any reason the investigator considers it advisable, the study drug can be withdrawn, but all such patients remain in the study and continue to receive full annual examinations and 6-month interim follow-up visits until the end of the study period. All the information obtained at every 6-month follow-up visit along with the information on cardiovascular events, adverse events, and investigations into discontinuation, if any, will be transmitted to the COPE Trial Data Center. Since patient withdrawal decreases the study's statistical power to detect