FKBP12.6-Mediated Stabilization of Calcium-Release Channel (Ryanodine Receptor) as a Novel Therapeutic Strategy Against Heart Failure

Masafumi Yano, MD, PhD; Shigeki Kobayashi, MD, PhD; Masateru Kohno, MD; Masahiro Doi, MD; Takahiro Tokuhisa, MD; Shinichi Okuda, MD; Masae Suetsugu, BS; Takayuki Hisaoka, MD, PhD; Masakazu Obayashi, MD, PhD; Tomoko Ohkusa, MD, PhD; Michihiro Kohn, MD, PhD; Masunori Matsuzaki, MD, PhD

Background—The development of heart failure is tightly correlated with a decrease in the stoichiometric ratio for FKBP12.6 binding to the ryanodine receptor (RyR) in the sarcoplasmic reticulum (SR). We report that a new drug, the 1,4-benzothiazepine derivative JTV519, reverses this pathogenic process. JTV519 is known to have a protective effect against Ca\(^{2+}\)/H\(^{+}\) overload–induced myocardial injury.

Methods and Results—Heart failure was produced by 4 weeks of rapid right ventricular pacing, with or without JTV519; SR were then isolated from dog left ventricular (LV) muscles. First, in JTV519-treated dogs, no signs of heart failure were observed after 4 weeks of chronic right ventricular pacing, LV systolic and diastolic functions were largely preserved, and LV remodeling was prevented. Second, JTV519 acutely inhibited both the FK506-induced Ca\(^{2+}\)/H\(^{+}\) leak from RyR in normal SR and the spontaneous Ca\(^{2+}\)/H\(^{+}\) leak in failing SR. Third, there was no abnormal Ca\(^{2+}\)/H\(^{+}\) leak in SR vesicles isolated from JTV519-treated hearts. Fourth, in JTV519-treated hearts, both the stoichiometry of FKBP12.6 binding to RyR and the amount of RyR-bound FKBP12.6 were restored toward the values seen in normal SR. Fifth, in JTV519-untreated hearts, RyR was PKA-hyperphosphorylated, whereas it was reversed in JTV519-treated hearts, returning the channel phosphorylation toward the levels seen in normal hearts.

Conclusions—During the development of experimental heart failure, JTV519 prevented the amount of RyR-bound FKBP12.6 from decreasing. This in turn reduced the abnormal Ca\(^{2+}\)/H\(^{+}\) leak through the RyR, prevented LV remodeling, and led to less severe heart failure. (Circulation. 2003;107:477-484.)

Key Words: sarcoplasmic reticulum ■ heart failure ■ calcium ■ ion channels ■ remodeling

Abnormal regulation of intracellular Ca\(^{2+}\) by the sarcoplasmic reticulum (SR) is the chief pathogenic mechanism underlying a number of the dysfunctions that occur in heart failure.\(^1,2\) In a dog model of pacing-induced heart failure, we previously found that a prominent abnormal Ca\(^{2+}\) leak occurs through ryanodine receptor (RyR), owing to a partial loss of RyR-bound FKBP12.6,\(^3\) in association with a decreased rate of Ca\(^{2+}\) release through the RyR.\(^4,5\) Removal of FKBP12.6 from RyR causes uncoupled channel gating in the RyR, which results in defective closure of these channels.\(^6,7\) In a study of the mechanism underlying the partial loss of FKBP12.6 from RyR, Marx et al\(^8\) demonstrated that hyperphosphorylation of RyR, mediated by PKA, causes dissociation of FKBP12.6 from RyR, and this in turn causes an increased sensitivity to Ca\(^{2+}\)-induced activation and defects in channel functions. These findings suggest that failing hearts lack the normal FKBP12.6-mediated channel regulation and that this is the major cause of the serious abnormality in the regulation of intracellular Ca\(^{2+}\) and the consequent cardiac dysfunctions seen in such hearts.

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One of the 1,4-benzothiazepine derivatives, JTV519, has been shown to have a protective effect against Ca\(^{2+}\)/H\(^{+}\) overload–induced myocardial injury, presumably by preventing the Ca\(^{2+}\) overload that results from increased release from the intracellular Ca\(^{2+}\) store.\(^9\) Hachida et al\(^10\) reported that the beneficial effects of this drug against ischemic myocardial injury appear to be correlated with the ability of the drug to inhibit the postschismic rise in intracellular calcium. In the present study, we investigated whether chronic administration of JTV519 might prevent the development of heart failure in a canine model by restoring the FKBP12.6-dependent regulation of the RyR.

Methods

Materials
Human recombinant FKBP12.6 (rFKBP12.6) and FKBP 12 (FKBP12) were produced in our laboratory. FK506 was provided...
Production of Pacing-Induced Heart Failure

In beagle dogs weighing 10 to 14 kg (Kitayama Labes Co. Ltd, Nagano, Japan), we induced heart failure by 28 days of rapid right ventricular (RV) pacing at 250 bpm, using an externally programmable miniature pacemaker (Medtronic Inc), as described previously.4 Then, with the dogs under anesthesia, we chronically implanted a 5F micromanometer (Millar) in the left ventricle (LV) through the apex for the measurement of LV pressure. Because we found that JTV519 (0.5 mg/kg) significantly improved LV relaxation in dogs with rapid RV pacing (~14% reduction in the time constant of LV pressure decay) for at least 1 hour when it was acutely infused (unpublished data), we continuously infused JTV519 (12 [0.5×24] mg/kg per day) by means of an infusion pump. At this dose, the plasma concentrations in dogs with 1 week (1w) and 4 weeks (4w) of pacing were 822.9±6.0 ng/mL and 823.9±3.31 ng/mL (n=4), respectively. To obtain the diastolic pressure–diameter relation, phenylephrine (2 to 10 µg/kg per minute) was infused intravenously for 10 minutes to increase LV pressure in 8 conscious dogs [4 dogs, JTV519(–); 4 dogs, JTV519(+)].

Preparation of SR Vesicles

We prepared SR vesicles essentially by the method of Kranias et al,11 with modifications that have been described elsewhere.4

Ca2+ Uptake and Ca2+ Leak Assays

Ca2+ uptake and the following Ca2+ leak assays were done as described previously.3 Ca2+ uptake function under different [Ca2+] was measured with the use of “Ca, as described previously.4

[3 H]dihydro-FK506 and [3 H]ryanodine Binding Assays

We performed [3 H]dihydro-FK506– and [3 H]ryanodine– (Dupont NEN) binding assays as described previously.3,4 We calculated the stoichiometry of FKBP per RyR directly from the ratio of the Bmax values for [3 H]dihydro-FK506 binding and [3 H]ryanodine binding.12

Site-Directed Labeling of RyR

We performed specific fluorescent labeling of RyR in SR vesicles by using the cleavable heterobifunctional cross-linking reagent SAED [sulfosuccinimidyl 3-((2-(7-azido-4-methylcoumarin-3-acetamido)ethyl) dithiopropionate from PIERCE], with polyly-
sine as a site-specific carrier, as described previously.\textsuperscript{3,13,14} Then, we monitored the time course of the FK506-induced changes in the fluorescence intensity (arbitrary units) of the RyR-bound methyl-coumarin-acetamido (MCA) probe under the same conditions as those used for the Ca\textsuperscript{2+}-leak assay as described previously.\textsuperscript{3}

**Immunoblot Analysis**

We performed immunoblot analyses for FKBP12.6 and SR Ca\textsuperscript{2+}-ATPase as previously described.\textsuperscript{3} As recommended by Marx et al,\textsuperscript{8} we achieved coimmunoprecipitation of FKBP12.6 from SR by using anti-RyR antibody (Oncogene Research Products) followed by immunoblotting with anti-FKBP12 (C-19) antibody\textsuperscript{15} (Santa Cruz Biotechnology). Specific antibodies against phosphoserine 16-phospholamban (PLB; Upstate biotech) and an epitope common to all PLB forms (PLB; Upstate biotech) were also used.

**Dissociation and Reconstitution of FKBP12.6**

We achieved dissociation of FKBP12.6 from, and its reconstitution into, SR vesicles by the method of Timerman et al,\textsuperscript{12} with slight modifications. Briefly, we preincubated SR vesicles (2 mg/mL) in imidazole homogenization medium (IHM) (5 mmol/L imidazole-Cl, pH 7.4, and 0.3 mol/L sucrose) containing 5 μmol/L FK506. We centrifuged samples to yield sedimentable and supernatant fractions. The pellet was resuspended in IHM buffer and is referred to as FKBP12.6-deficient SR. We also attempted to achieve reconstitution of FKBP12.6 or FKBP12 into failing SR vesicles by mixing rFKBP12.6 or rFKBP12 (1 μg/mL) with failing SR vesicles (2 mg/mL) at room temperature for 30 minutes in the presence or absence of 1 μmol/L JTV519.

**Statistics**

We used a 1-way or 2-way ANOVA to compare data between groups. When we identified a significant trend by the \( F \) test, we used Scheffé’s post hoc test to compare the data. Data are expressed as mean±SD. We accepted a probability value <0.05 as statistically significant (*\( P < 0.05 \), **\( P < 0.01 \) versus normal or prepacing, #\( P < 0.05 \), ##\( P < 0.01 \) versus without JTV519 or JTV519-untreated. +\( P < 0.05 \), ++\( P < 0.01 \) versus 1w of pacing).
Results

Hemodynamic Data and Echocardiographic Assessment

In JTV519-treated dogs with chronic RV pacing, both systolic and diastolic functions were largely preserved, and heart failure developed in none of these dogs (Table 1 and Figure 1a). We obtained representative diastolic pressure (P)–diameter (D) relations during phenylephrine infusion (Figure 1b). These demonstrate that in the JTV519-untreated dog, the curve shifted to the right as the pacing period increased, indicating the development of LV remodeling (Figure 1b). In contrast, there was no such shift in the JTV519-treated dog. Plasma norepinephrine, angiotensin II, and atrial natriuretic peptide were all higher after rapid chronic pacing than in normal dogs without RV pacing. Chronic administration of JTV519 significantly reduced the levels of all these neurohormonal factors in the paced-heart dogs (Table 1). These data indicate that in the JTV519-treated dogs, there were no signs of heart failure after chronic RV pacing.

Restoration of Normal Mode of FKBP12.6-Mediated Stabilization of RyR

JTV519 almost completely inhibited the FK506-induced Ca\(^{2+}\) leak in normal SR vesicles and also the spontaneous Ca\(^{2+}\) leak in failing SR vesicles (Figure 2a). Diltiazem partially inhibited both Ca\(^{2+}\) leaks at slightly higher concentrations than JTV519, and neither nifedipine nor verapamil affected either type of Ca\(^{2+}\) leak (Figure 2b). In JTV519-treated SR vesicles from 1w- and 4w-paced hearts, a spontaneous Ca\(^{2+}\) leak was not observed (Figure 2c).

The addition of FK506, after completion of Ca\(^{2+}\) uptake, led to an increase in MCA fluorescence in normal SR vesicles but virtually no change in MCA fluorescence in failing SR vesicles (Figure 2d). We proposed previously that an MCA fluorescence change of the type shown in Figure 2d reflects the time course of the conformational change in RyR produced by the FK506-induced dissociation of FKBP12.6 from the RyR. JTV519 completely inhibited the FK506-induced change in MCA fluorescence in normal SR vesicles (Figure 2d). Interestingly, JTV519 decreased the level of MCA fluorescence in failing SR vesicles, although there was no change at all in normal SR vesicles. These results suggest the following. In failing SR vesicles, the RyR are in a state of high MCA fluorescence (corresponding to the FKBP-deficient RyR state), and the binding of JTV519 to the RyR produced a low MCA fluorescence state equivalent to a low Ca\(^{2+}\)-leak state (and corresponding to the FKBP-bound state).

The above view is supported by the following experiment (Figure 3). In normal SR, dissociation of FKBP12.6 by 30 \(\mu\)mol/L FK506 from normal SR and its effect on baseline MCA fluorescence level and Ca\(^{2+}\) leak. IP indicates fraction immunoprecipitated from SR using anti-RyR antibody followed by immunoblotting with anti-FKBP12 antibody. S indicates supernatant containing soluble FKBP-FK506 complex; P, pellet. b, Inhibitory effect of JTV519 (1 \(\mu\)mol/L) on dissociation of FKBP12.6 from RyR by various concentrations of FK506 in normal SR. c, Inhibitory effect of JTV519 (1 \(\mu\)mol/L) on dissociation of FKBP12.6 from RyR induced by cAMP-dependent (0.3 \(\mu\)mol/L) PKA phosphorylation of RyR in normal SR. PKI indicates protein kinase A inhibitor. d, Reconstitution of rFKBP12.6 (1 \(\mu\)g/mL) into failing SR vesicles (2 mg/mL) and its effect on baseline MCA fluorescence level and Ca\(^{2+}\) leak. Both the immunoblotting of RyR and PKA back-phosphorylation of the RyR were also shown. Back-phosphorylation of the immunoprecipitated RyR2 was initiated with Mg-ATP containing 10% [\(^{32}\)P]ATP (NEN Life Sciences) in the presence of PKA (5 U). Note that the MCA fluorescence (net value, in arbitrary units: labeled SR minus unlabeled SR) increased as SR became FKBP12.6-deficient but decreased as SR bound FKBP12.6 again.
FKBP12.6 from RyR to the lower concentrations of FK506 (Figure 3b). Also, JTV519 inhibited the dissociation of FKBP12.6 from RyR induced by cAMP-dependent PKA phosphorylation (Figure 3c). JTV519 did not directly affect the phosphorylation level of RyR on reconstitution, as confirmed by back-phosphorylation (Figure 3d). The reconstitution of failing SR with FKBP12.6 occurred with significantly greater efficiency in the presence of JTV519, indicating that the binding of FKBP12.6 to RyR was strengthened by JTV519 (Figure 3d). The actual values obtained for MCA fluorescence showed that MCA fluorescence was lower in the normal SR than in the failing SR (Figure 3, a and d, boxes). On dissociation of FKBP12.6 from normal SR, the MCA fluorescence increased, whereas on addition of FKBP12.6 to failing SR, it decreased.

Effect of JTV519 on PKA Phosphorylation of RyR and the Amount of FKBP12.6

In JTV519-treated SR vesicles, the $B_{\text{max}}$ values for $[^{3}H]$dihydro-FK506 binding were significantly larger than the corresponding values for JTV519-untreated vesicles, though still significantly less than those for normal SR vesicles (Figure 4a and Table 2). The molar ratio was larger for JTV519-treated vesicles (3.58±0.74) than for JTV519-untreated vesicles (1.33±0.46) and very close to that obtained for normal SR vesicles (3.55±0.65) (Table 2). In the JTV519-untreated SR vesicles, RyR was PKA-hyperphosphorylated, whereas it was reversed in the...
TABLE 2. \[^{3}H\]dihydro-FK506 and \[^{3}H\]ryanodine binding to SR vesicles

<table>
<thead>
<tr>
<th></th>
<th>[^{3}H]dihydro-FK506 Binding</th>
<th>[^{3}H]ryanodine Binding</th>
<th>Stoichiometry (FKBP/RyR)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>(B_{\text{max}}, \text{pmol/mg})</td>
<td>(K_{d}, \text{nmol})</td>
<td>(B_{\text{max}}, \text{pmol/mg})</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>No pacing ((n=5))</td>
<td>7.93±0.52</td>
<td>8.71±2.92</td>
<td>2.28±0.34</td>
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<tr>
<td>JTV519 untreated</td>
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<tr>
<td>Pacing, 1w ((n=4))</td>
<td>4.70±0.13†</td>
<td>6.32±0.67</td>
<td>1.69±0.26*</td>
</tr>
<tr>
<td>Pacing, 4w ((n=5))</td>
<td>1.66±0.49††</td>
<td>4.93±2.22</td>
<td>1.28±0.26††</td>
</tr>
<tr>
<td>JTV519 treated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pacing, 1w ((n=4))</td>
<td>6.74±0.46§</td>
<td>9.05±1.60§</td>
<td>1.83±0.14</td>
</tr>
<tr>
<td>Pacing, 4w ((n=5))</td>
<td>4.97±0.92§§</td>
<td>11.92±4.27§§</td>
<td>1.43±0.31†</td>
</tr>
</tbody>
</table>

The parameters for \[^{3}H\]dihydro-FK506 binding and \[^{3}H\]ryanodine binding to cardiac muscle SR fractions were determined by Scatchard analysis. \(B_{\text{max}}\) indicates the maximal number of binding sites; \(K_{d}\) dissociation constant.

\*\(P<0.05\), \(†P<0.01\) vs prepacing.

\‡\(P<0.05\), \§\(P<0.01\) vs JTV519 untreated.

\|\(P<0.01\) vs 1w of pacing.

Figure 5. a, Acute effect of JTV519 (1 \(\mu\)mol/L) on \(\text{Ca}^{2+}\) uptake function (free[\(\text{Ca}^{2+}\)] = 0.3 \(\mu\)mol/L or 1 \(\mu\)mol/L) in normal and failing SR vesicles. b, \(\text{Ca}^{2+}\) uptake function in normal, JTV519-untreated failing, and JTV519-treated failing SR vesicles. c, Amount of SR \(\text{Ca}^{2+}\) ATPase in SR vesicles: lane 1, normal; lane 2, 1w of pacing; lane 3, 1w of pacing with JTV519; lane 4, 4w of pacing; lane 5, 4w of pacing with JTV519. d, Representative Western blot analysis of Ser16-phosphorylated PLB (p-PLB) and total PLB (t-PLB), and densitometric analysis of the Western blot. PLB (H) indicates high-molecular-weight PLB; PLB (L), low-molecular-weight PLB. The p-PLB values [(sum of PLB(H) and PLB(L), in arbitrary units)] were normalized by t-PLB values [(sum of PLB(H) and PLB(L), in arbitrary units)]. Data are mean±SD. \*\(P<0.01\) vs normal SR. \#\(P<0.05\) vs 1w of pacing without JTV519.
JTV519-treated vesicles, returning the channel phosphorylation toward the levels seen in normal hearts (Figure 4b).

The amount of SR-associated FKBP12.6 was significantly larger in JTV519-treated vesicles than in JTV519-untreated SR in both the 1w and 4w RV pacing groups (Figure 4c).

Chronic treatment with JTV519 improves Ca^{2+} uptake function, and a phosphorylation level of PLB JTV519 had no acute effect on Ca^{2+} uptake function in both vesicles (Figure 5a). After 4w of rapid RV pacing, the decreases in both SR Ca^{2+} uptake and the amount of SR Ca^{2+}-ATPase were greater in JTV519-untreated SR vesicles than in JTV519-treated SR vesicles (Figure 5, b and c). Figure 5d compares the level of Ser16-phosphorylated PLB (p-PLB) and total PLB (t-PLB) in the SR vesicles. There was no change in the level of total PLB among all groups, but there was a significant decrease in the basal level of phosphorylated PLB in failing SR vesicles. In JTV-treated SR vesicles, the level of phosphorylated PLB was restored back toward normal.

Discussion

In a canine model of heart failure, it has been recently demonstrated that \(\beta\)-adrenergic receptor blocker indeed corrects the defective interaction of FKBP12.6 with RyR triggered by the PKA-mediated hyperphosphorylation of RyR (restoration of stoichiometry of the RyR2 macromolecular complex,\(^{16}\) normalization of single-channel function \(\text{RyR,}^{16}\) and prevention of \(\text{Ca}^{2+}\) leak from \(\text{RyR}^{17}\)). This study further strengthened the possibility that FKBP12.6-mediated stabilization of RyR can indeed be a new therapeutic strategy against heart failure.

The major findings of this study are that JTV519 prevented dissociation of FKBP12.6 from RyR, hence inhibiting conformational change in RyR and the subsequent abnormal \(\text{Ca}^{2+}\) leak, in the early developmental stage of heart failure, thereby leading to less severe heart failure. Since JTV519 inhibited the spontaneous \(\text{Ca}^{2+}\) leak in failing SR vesicles, from which FKBP12.6 was already partially lost, JTV519 would seem to exert an FKBP12.6-like channel-stabilizing effect directly.

JTV519, a benzothiazepine derivative, shares an analogous chemical structure with the dihydropyridine-binding \(\text{Ca}^{2+}\)-channel blocker diltiazem.\(^9\) Indeed, Kimura et al\(^{18}\) found that JTV519 (1 \(\mu\)mol/L) decreases the inward \(\text{Ca}^{2+}\)-current by \(\approx 20\%\). We therefore compared the effects of JTV519 on \(\text{Ca}^{2+}\) leak in normal and failing SR vesicles with those of three different \(\text{Ca}^{2+}\) antagonists (diltiazem, verapamil, and nifedipine). Neither nifedipine nor verapamil inhibited the \(\text{Ca}^{2+}\) leak in either type of RyR, but, interestingly, diltiazem did partially inhibit these leaks, albeit at higher concentrations than JTV519. Presumably, the common chemical structure shared by JTV519 and diltiazem may lead to them having a similar RyR-stabilizing effect, and this then leads to an inhibition of both types of \(\text{Ca}^{2+}\) leak. Because JTV519 has a capability of binding with annexin V, a component of the cytoskeleton structure after entering the cell,\(^9,19\) it is suggested that it also interacts directly with RyR2 after passing through the plasma membrane in vivo.

The improvement in LV relaxation induced by JTV519 may also be mediated through an enhancement of SR \(\text{Ca}^{2+}\)-ATPase activity because both \(\text{Ca}^{2+}\) uptake and the amount of \(\text{Ca}^{2+}\)-ATPase were increased at 4w of RV pacing (Figure 5, b and c), in association with the increase in the basal level of Ser16 phosphorylated PLB. In both normal and failing SR vesicles, JTV519 had no direct effect on PLB-phosphorylation induced by cAMP in vitro (unpublished data). As mentioned by Marks et al,\(^{20}\) PKA phosphorylation of specific targets within cardiomyocyte appears to be compartmentalized such that some proteins (eg, RyR) are PKA-hyperphosphorylated in failing hearts, whereas other \(\text{Ca}^{2+}\) handling proteins (eg, PLB) are hypophosphorylated in the same hearts.

In conclusion, the important novel finding made in this study of a canine model of heart failure is that chronic administration of JTV519 improved LV systolic and relaxation functions and led to less severe heart failure. Our results strongly suggest that this cardioprotective effect is produced by restoring the normal FKBP12.6-mediated stabilization of the RyR \(\text{Ca}^{2+}\) channel, defectiveness of which is the major cause of a variety of abnormal functions in the failing heart (Figure 6).

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References


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