Propranolol Prevents the Development of Heart Failure by Restoring FKBP12.6-Mediated Stabilization of Ryanodine Receptor

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Background—In heart failure, protein kinase A–mediated hyperphosphorylation of ryanodine receptors (RyRs) in sarcoplasmic reticulum (SR) causes dissociation of FKBP12.6 from RyRs. This results in an abnormal Ca2+ leak through RyRs, possibly leading to cardiac dysfunction. In the present study, we assess whether β-blockers can correct this defect in RyR in tachycardia-induced heart failure and thereby improve cardiac function.

Methods and Results—SRs were isolated from dog left ventricular muscles (normal group, 4 weeks of rapid right ventricular pacing with or without propranolol [P(+)] or P(−))]. End-diastolic and end-systolic diameters both increased less in P(+) than P(−), associated with a smaller decrease in fractional shortening in P(+). In SR from P(−), a prominent Ca2+ leak was observed, and FK506 (which dissociates FKBP12.6 from RyR) did not induce an additional Ca2+ leak. However, there was no appreciable Ca2+ leak in SR from P(+), although FK506 induced a Ca2+ leak as in normal SRs. In SR from P(+), an FK506-induced conformational change in RyR, which was virtually absent in SR from P(−), was observed as in normal SRs. Both the stoichiometry of FKBP12.6 versus RyR, assessed by [3H]FK506 binding assays, and the protein expression of FKBP12.6, assessed by Western blot analysis, were restored by propranolol toward the levels seen in normal SRs.

Conclusions—Low-dose propranolol corrects the defective interaction of FKBP12.6 with RyR (restoration of RyR conformational change and prevention of Ca2+ leak from RyR), apparently resulting in an attenuation of intracellular Ca2+ overload and hence preventing the development of left ventricular remodeling in heart failure. (Circulation. 2002;105:1374-1379.)

Key Words: β-blocker ■ heart failure ■ sarcoplasmic reticulum ■ calcium

An abnormal regulation of intracellular Ca2+ by sarcoplasmic reticulum (SR) has been shown to be involved in the mechanism underlying the contractile and relaxation dysfunctions in heart failure. Several investigators have demonstrated that in cardiac hypertrophy or failure, Ca2+ uptake by the SR is decreased in conjunction with a decreased density of Ca2+ ATPase.1,2 Within the last few years, an altered function of the SR Ca2+ release channel (ryanodine receptor [RyR]) has also been shown to contribute to cardiac dysfunction in heart failure.3-5 As described in our previous study,6 in a dog model of pacing-induced heart failure, a prominent abnormal Ca2+ leak occurs through the RyR. This is attributable to a partial loss of RyR-bound FKBP12.6 and the resultant conformational change in the RyR. Presumably, this abnormal Ca2+ leak causes an intracellular Ca2+ overload, which in turn leads to diastolic and systolic dysfunctions. We also found that in the failing heart, polylsine-induced Ca2+ release from SR vesicles was decreased, owing to an impaired gating function of the RyR,7 and this too is ascribable to a defective FKBP12.6-RyR interaction.8 Removal of FKBP12.6 from RyR causes uncoupled channel gating in the RyR, resulting in defective closure of these channels.9,10 With regards to the mechanism responsible for the partial loss of FKBP12.6 from the RyR, Marx et al11 demonstrated that RyR hyperphosphorylation, mediated by protein kinase A (PKA), causes dissociation of FKBP12.6 from RyR, which in turn causes an increased sensitivity to Ca2+-induced activation and defective channel functions. These findings suggest that failing hearts lack FKBP-mediated channel regulation and that this is the major cause of the serious abnormality in their regulation of intracellular Ca2+ and their observed cardiac dysfunctions.
A common finding in patients with heart failure is that a hyperadrenergic state and elevated levels of circulating catecholamines are markers for increased risk of mortality. Moreover, clinical trials have shown that treatment with β-blockers restores cardiac function and reduces rate of mortality in patients with heart failure. The experimental literature also suggests that the alterations in biology and contractility seen in the failing cardiac myocyte can be reversed by β-blockers. However, the actual mechanism responsible for these beneficial effects of β-blockers has not been fully elucidated. In the present study, we used a dog model of pacing-induced heart failure to investigate whether β-blockers would inhibit PKA-mediated RyR hyperphosphorylation, prevent the dissociation of FKBP12.6 from RyR, inhibit the abnormal Ca²⁺ leak through RyR, and thereby restore cardiac function.

Methods

Fluo-3 was obtained from Molecular Probes, and SAED was from Pierce. [³H]ryanodine, [³H]dihydro-FK506, and [γ-³²P]ATP were purchased from Dupont NEN. Anti-FKBP12 (C-9) antibody, which cross-reacts with FKBP12.6, was purchased from Santa Cruz Biotechnology. Anti-RyR antibody and Anti-SERCA2 antibody were obtained from Oncogene Research Products and Affinity Bioreagents Inc, respectively. Human recombinant FKBP12.6 was produced in our laboratory. FK506 was provided by Fujisawa Pharmaceutical Co Ltd (Osaka, Japan).

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. Then, under anesthesia, we chronically implanted a 5-F micromanometer in the left ventricle (LV) via the apex for the measurement of LV pressure, and we placed a pair of crystals (5 MHz, 2 mm in diameter) on the endocardium of the anterior and posterior walls perpendicular to the long axis of the LV, midway between the apex and the base of the heart. After allowing a recovery period of 1 week, we measured LV pressure and recorded two-dimensional echocardiograms at the level of the head of the papillary muscle in the conscious state, ~1 hour after the termination of rapid RV pacing. The protocols used were in accordance with guidelines set by the Animal Ethics Committee of Yamaguchi University School of Medicine.

To determine the dose of propranolol to be used for chronic administration, we evaluated the concentration-dependent effect of propranolol on hemodynamic parameters in normal conscious dogs (Figure 1). Propranolol was continuously infused for 1 day, starting at a rate of 0.05 mg/kg per day and increasing incrementally to 2 mg/kg per day. A recovery period of 1 day was allowed before each incremental increase. Propranolol (2 mg/kg per day) was found to significantly decrease maximal inotropic response induced by isoproterenol (0.80 μg · kg⁻¹ · min⁻¹ IV) (Figure 1). In our first dog, we continuously infused this dose of propranolol simultaneously with the initiation of RV pacing. However, after 1 day of this infusion, the dog died of severe cardiac dysfunction. Therefore, in the next dog, we continuously infused 0.5 mg/kg per day of propranolol, by which means a 17% decrease in the inotropic response had been obtained in normal dogs (Figure 1). However, after infusion for 3 days with pacing, that dog also died from the same cause. We then decided to try a much lower dose, 0.05 mg/kg per day, for chronic administration in the following experiments. At this dose, neither the baseline max dP/dt nor the isoproterenol-induced inotropic response was decreased, although baseline heart rate was decreased significantly by (15%) in normal conscious dogs (Figure 1). Thus, a negative chronotropic effect, but not a negative inotropic effect, was elicited at this dose.

To obtain LV diastolic pressure–diameter relationship over a wide range of diastolic pressures, phenylephrine (2 to 10 μg · kg⁻¹ · min⁻¹) was infused intravenously for 10 minutes to increase LV pressure in 4 conscious dogs (4 dogs, propranolol –; 4 dogs, propranolol +).

Preparation of SR Vesicles

We prepared SR vesicles essentially by the method of Kranias et al, with the modifications described elsewhere.

Ca²⁺ Uptake and Leak Assays

We first incubated SR vesicles (0.2 mg/mL) in 0.5 mL of solution containing 0.15 mol/L potassium glutonate, 1 mmol/L MgCl₂, 0.2 mmol/L EGTA-calcium buffer (free [Ca²⁺]), 0.3 μmol/L, 10 mmol/L Na₃, and 20 mmol/L MOPS, pH 6.8. Ca²⁺ uptake was initiated by the addition of 0.5 mmol/L ATP into the cuvette. After the Ca²⁺ uptake had reached a plateau, we added 1 μmol/L thapsigargin to inhibit SR Ca²⁺-ATPase activity with or without FK506 (30 μmol/L) and monitored the resultant Ca²⁺ leak. We monitored the time course of Ca²⁺ uptake spectrophotometrically (F-2000, Hitachi) using fluo-3 as a Ca²⁺ indicator (excitation 350 nm, emission 530 nm), as described previously. The magnitude of the Ca²⁺ leak was taken as the value obtained 60 seconds after the addition of thapsigargin, and it was expressed as a percentage of the preceding Ca²⁺ uptake.

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

We performed [³H]dihydro-FK506 and [³H]ryanodine binding assays as described previously. We determined the density of high affinity [³H]ryanodine binding sites in SR vesicles by Scatchard analysis of [³H]ryanodine binding isotherms, as described previously.

Site-Directed Fluorescent Labeling of the RyR

We performed specific fluorescent labeling of RyR in SR vesicles using the cleavable hetero-bifunctional cross-linking reagent sulfo-succinimidyl 3-(2-(7-azido-4-methylcoumarin-3-acetamido) ethyl) dithio/propiionate (SAED), with polylysine as a site-specific carrier, as described previously. We monitored the time course of the FK506-induced changes in the fluorescence intensity (arbitrary units) of the RyR-bound methyleucomarin-acetate (MCA) probe (excitation 335 nm, emission 450 nm) under the same conditions as those used for the Ca²⁺ leak assay (except that there was no fluo-3 in
the reaction solution). For this monitoring, we used a fluorescence spectrophotometer (F-2000; Hitachi).

**Immunoblot Analysis**

We performed immunoblot analyses for FKBP12.6 and SR Ca\(^{2+}\)-ATPase as previously described. By using the method by Marx et al., we achieved communoprecipitation of FKBP12.6 from SR using anti-RyR antibody followed by immunoblotting with anti-FKBP12 antibody. PKA-mediated phosphorylation of immunoprecipitated RyR was assessed with back-phosphorylation using [\(^{32}\)P]ATP, as previously described.

**Statistics**

Intragroup comparisons were made by paired \(t\) test. Intergroup analysis was performed by ANOVA with a post hoc Schiff’s test. Data are expressed as mean \(\pm\) SD. We accepted \(P<0.05\) as statistically significant.

**Results**

In the propranolol-treated dogs with chronic RV pacing, both systolic and diastolic functions were preserved, and none of these dogs developed heart failure (Table 1 and Figure 2A). The representative diastolic pressure-diameter relationship obtained during phenylephrine infusion shown in Figure 2B revealed that in the propranolol-untreated dog, the diastolic pressure-diameter relationship curve shifted to the right after a 4-week period of pacing, indicating the development of LV remodeling. In contrast, there was a much less pronounced shift in the propranolol-treated dog (Figure 2B). The plasma contents of norepinephrine and atrial natriuretic peptide and angiotensin II were higher in dogs with rapid chronic RV pacing than in normal dogs. Chronic administration of propranolol during pacing significantly reduced these levels (Figure 2C). These data indicate that our propranolol-treated dogs showed no signs of heart failure despite chronic RV pacing.

Addition of 1 \(\mu\)mol/L thapsigargin to normal SR vesicles produced a small Ca\(^{2+}\) leak, whereas addition of 30 \(\mu\)mol/L FK506 together with 1 \(\mu\)mol/L thapsigargin produced a much more pronounced leak (Figure 3). In contrast, in failing (propranolol-untreated) SR vesicles, addition of thapsigargin alone produced a prominent Ca\(^{2+}\) leak, but addition of FK506 produced no additional increase. In SR vesicles from paced, propranolol-treated dogs, a spontaneous Ca\(^{2+}\) leak was not observed, and FK506 had the same effect as in normal SR (that is, it greatly increased the Ca\(^{2+}\) leak).

In normal SR vesicles, the addition of FK506 after Ca\(^{2+}\) uptake had plateaued induced an increase in MCA fluorescence at a faster rate than Ca\(^{2+}\) leak seen in the same SR vesicles, but it produced virtually no increase in MCA fluorescence in failing (propranolol-untreated) SR vesicles (Figure 4). In propranolol-treated SR vesicles, FK506 in-

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**TABLE 1.** Hemodynamic Data

<table>
<thead>
<tr>
<th>Condition</th>
<th>HR, bpm</th>
<th>LVSP, mm Hg</th>
<th>LVEDP, mm Hg</th>
<th>((±))dP/dt, mm Hg/s</th>
<th>(\tau), ms</th>
<th>LVEDD, mm</th>
<th>LVESD, mm</th>
<th>LVFS, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propranolol-untreated (n=6)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Prepping</td>
<td>127±25</td>
<td>122±18</td>
<td>7.9±4.7</td>
<td>2966±410</td>
<td>19.5±4.6</td>
<td>31.1±1.7</td>
<td>19.2±1.0</td>
<td>38.1±2.9</td>
</tr>
<tr>
<td>4w-pacing</td>
<td>136±11</td>
<td>109±4</td>
<td>31.8±4.0†</td>
<td>1391±215†</td>
<td>43.8±13.6†</td>
<td>41.2±1.7</td>
<td>36.1±1.8</td>
<td>12.2±3.4†</td>
</tr>
<tr>
<td>Propranolol-treated (n=6)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Prepping</td>
<td>117±13</td>
<td>137±6</td>
<td>9.5±3.5</td>
<td>3276±323</td>
<td>19.0±2.8</td>
<td>31.0±1.7</td>
<td>19.3±1.6</td>
<td>37.7±2.7</td>
</tr>
<tr>
<td>4w-pacing</td>
<td>120±11</td>
<td>111±3†</td>
<td>22.2±6.8†‡</td>
<td>1555±237†</td>
<td>29.3±6.4‡</td>
<td>36.5±1.5‖§</td>
<td>30.0±1.8‖§</td>
<td>19.2±3.4‖§</td>
</tr>
</tbody>
</table>

HR indicates heart rate; LVSP, left ventricular peak-systolic pressure; LVEDP, left ventricular end-diastolic pressure; \((±)\)dP/dt, peak \((±)\)dP/dt of LV pressure; \(\tau\), time constant of left ventricular pressure decay during isovolumic relaxation period; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; and FS, fractional shortening (LVEDD-LVESD/LVEDD x 100). Data represent mean \(±\) SD. *\(P<0.05\), †\(P<0.01\) vs prepacing; ‡\(P<0.05\), §\(P<0.01\) vs propranolol-untreated.
Additional changes in RyR produced by the FK506-induced fluorescence changes reflect the time course of conformational changes in SR vesicles. Changes in MCA fluorescence are expressed as \( F_0 \) where \( F_0 \) indicates before addition of FK506 (baseline) and \( F \) indicates after addition of FK506.

Figure 3. A, Representative time courses of Ca\(^{2+}\) uptake and the ensuing Ca\(^{2+}\) leak from SR vesicles obtained from normal and 4w-paced hearts. Note that after propranolol treatment, the spontaneous Ca\(^{2+}\) leak seen in failing SR vesicles disappeared. Note also that FK506 enhanced the Ca\(^{2+}\) leak in the paced, propranolol-treated dog and in the normal dog but not in the paced, propranolol-untreated dog. B, Comparison of spontaneous and FK506-induced Ca\(^{2+}\) leaks in SR vesicles from normal, paced propranolol-untreated, and paced propranolol-treated groups.

Figure 4. A, Effect of FK506 on representative time courses of the change in MCA fluorescence during Ca\(^{2+}\) leak in SR vesicles. Note that in propranolol-treated failing SR vesicles, a prominent MCA fluorescence change was observed, as in normal SR vesicles. B, Comparison of FK506-induced MCA fluorescence change in normal SR vesicles and propranolol-untreated and -treated failing SR vesicles. Changes in MCA fluorescence are expressed as \( \frac{F - F_0}{F_0} \times 100 \)%, where \( F_0 \) indicates before addition of FK506 (baseline) and \( F \) indicates after addition of FK506.
Recently we found that the development of the heart failure induced by chronic RV pacing can be completely prevented by restoring FKBP12.6-mediated stabilization of RyR using the cardioprotective reagent JTV519 (unpublished data, 2001). Taken together with the recent finding that in heart failure, PKA-mediated hyperphosphorylation of RyR leads to a defective FKBP12.6-mediated channel regulation of RyR, our results make it very likely that the mechanism by which propranolol improves cardiac function and prevents LV remodeling, without a change in Ca\textsuperscript{2+}/H\textsuperscript{1001} uptake function, depends on an inhibition of Ca\textsuperscript{2+} leak through RyR that results from a defective interaction of FKBP12.6 and RyR. The present finding that treatment with propranolol reversed the phosphorylation of RyR in conjunction with a reassociation of FKBP12.6 back to RyR supports the above view. Recently, Reiken et al\textsuperscript{24} reported that the \beta\textsuperscript{-}selective blocker metoprolol reversed PKA-mediated hyperphosphorylation of RyR\textsubscript{2}, restored the stoichiometry of RyR\textsubscript{2} macromolecular complex, and normalized single-channel function in a canine model of heart failure. We confirmed the beneficial effect of \beta\textsuperscript{-}blockers on RyR channel function using Ca\textsuperscript{2+}/H\textsuperscript{1001} leak and site-directed fluorescent assays. This effect seems to be exerted as a class effect of \beta\textsuperscript{-}blockers, because similar results were drawn regardless of the selectivity of the \beta\textsuperscript{-}blocker (\beta\textsuperscript{1}-selective or nonselective).

In heart failure, the contractile dysfunction that develops within myocytes during the process of LV remodeling is likely to involve other factors besides alterations in the excitation-contraction coupling process,\textsuperscript{25} ie, progressive loss of myofilaments from cardiac myocytes\textsuperscript{26} or alterations in cytoskeletal proteins, as well as desensitization of \beta\textsuperscript{-}adrenergic signaling.\textsuperscript{27} Resensitization of the \beta\textsuperscript{-}adrenergic

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TABLE 2. [\textsuperscript{3}H]dihydro-FK506 and [\textsuperscript{3}H]ryanodine Binding to SR Vesicles

<table>
<thead>
<tr>
<th></th>
<th>[\textsuperscript{3}H]dihydro-FK506 Binding</th>
<th>[\textsuperscript{3}H]ryanodine Binding</th>
<th>Stoichiometry (FKBP/RyR)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bmax, pmol/mg</td>
<td>Kd, nmol/L</td>
<td>Bmax, pmol/mg</td>
</tr>
<tr>
<td>Normal (n=5)</td>
<td>7.92±0.52</td>
<td>8.09±2.57</td>
<td>2.23±0.32</td>
</tr>
<tr>
<td>Propranolol-untreated</td>
<td>1.49±0.30†</td>
<td>4.59±2.09*</td>
<td>1.32±0.24†</td>
</tr>
<tr>
<td>Propranolol-treated (n=5)</td>
<td>3.38±0.38‡</td>
<td>7.03±3.00</td>
<td>1.44±0.21‡</td>
</tr>
</tbody>
</table>

The parameters for [\textsuperscript{3}H]dihydro-FK506 binding and [\textsuperscript{3}H]ryanodine binding to cardiac muscle SR fractions were determined by Scatchard analysis. The ratio of the Bmax values for [\textsuperscript{3}H]dihydro-FK506 and [\textsuperscript{3}H]ryanodine binding provides an estimate of the stoichiometry of FKBP per RyR.

Normal group indicates unpaced; propranolol-treated and -untreated groups, 4w-paced; Bmax, maximal number of binding sites; and Kd, dissociation constant. Data represent mean±SD. *P<0.05, †P<0.01 vs normal; ‡P<0.05, §P<0.01 vs propranolol-untreated.
In conclusion, in a canine model of heart failure, low-dose chronic propranolol treatment corrected the defective interaction of FKBP12.6 with RyR (restoration of RyR conformation), which may be different in other species, including human hearts. Therefore, the findings in the present study may not necessarily be converted to human hearts.

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