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 Ca\(^{2+}\) leak through RyRs, possibly leading to cardiac dysfunction. In the present study, we assess whether \(\beta\)-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 Ca\(^{2+}\) leak was observed, and FK506 (which dissociates FKBP12.6 from RyR) did not induce an additional Ca\(^{2+}\) leak. However, there was no appreciable Ca\(^{2+}\) leak in SR from P(+), although FK506 induced a Ca\(^{2+}\) 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 \([\text{H}]\)FK506 and \([\text{H}]\)ryanodine 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 Ca\(^{2+}\) leak from RyR), apparently resulting in an attenuation of intracellular Ca\(^{2+}\) overload and hence preventing the development of left ventricular remodeling in heart failure. (Circulation. 2002; 105:1374-1379.)

Key Words: \(\beta\)-blocker ■ heart failure ■ sarcoplasmic reticulum ■ calcium

An abnormal regulation of intracellular Ca\(^{2+}\) 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, Ca\(^{2+}\) uptake by the SR is decreased in conjunction with a decreased density of Ca\(^{2+}\) ATPase.1,2 Within the last few years, an altered function of the SR Ca\(^{2+}\) 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 Ca\(^{2+}\) 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 Ca\(^{2+}\) leak causes an intracellular Ca\(^{2+}\) overload, which in turn leads to diastolic and systolic dysfunctions. We also found that in the failing heart, polylysine-induced Ca\(^{2+}\) release from SR vesicles was decreased, owing to an impaired gating function of the RyR.7 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 Ca\(^{2+}\)–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 Ca\(^{2+}\) 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

Flu-3 was obtained from Molecular Probes, and SAED was from Pierce. [¹H]Ryanodine, [¹H]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 micrometer needle into 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. After the termination of rapid RV pacing, we performed a series of interventions to restore cardiac function, as 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 mmol/L NaCl, 10 mmol/L Na₂SO₄, and 20 mmol/L MOPS, pH 6.8. Ca²⁺ uptake was initiated by the addition of 0.5 mmol/L ATP into the cuvette. The Ca²⁺ uptake had reached a plateau, we added 1 mmol/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 (Hitachi) using fluo-3 as a Ca²⁺ indicator (excitation 480 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.

[¹H]Dihydro-FK506 and [¹H]Ryanodine Binding Assays

We performed [¹H]dihydro-FK506 and [¹H]ryanodine binding assays as described previously. We determined the density of high affinity [¹H]ryanodine binding sites in SR vesicles by Scatchard analysis of [¹H]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)propionate (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 methyllumarin-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

Figure 1. Concentration-dependent effect of propranolol on resting and isoproterenol-stimulated hemodynamic in normal conscious dogs. HR indicates heart rate; LVSPSP, left ventricular peak-systolic pressure; and (+) dP/dt, peak (+) dP/dt of LV pressure. Isoproterenol (0.80 kg⁻¹ min⁻¹ IV) was used to produce inotropic response. Data represent mean±SD obtained from 4 dogs. *P<0.05, **P<0.01 vs control.
TABLE 1. Hemodynamic Data

<table>
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<tr>
<th></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>
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<td>4w-pacing</td>
<td>136±11</td>
<td>109±4</td>
<td>31.8±4.0‡</td>
<td>1391±215†</td>
<td>43.6±13.6†</td>
<td>41.2±1.7†</td>
<td>36.1±1.8†</td>
<td>12.2±3.4†</td>
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<tr>
<td>Preparing</td>
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<td>137±6</td>
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<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×100). Data represent mean±SD. *\(P<0.05\), †\(P<0.01\) vs preparing; ‡\(P<0.05\), §\(P<0.01\) vs propranolol-untreated.

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±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-

Figure 2. Effects of propranolol on cardiac function and neurohormonal factors. A, Representative M-mode echocardiogram. Note that LV end-diastolic and -systolic diameters were each smaller in propranolol-treated dog than in propranolol-untreated dog (both 4-week paced). B, Representative diastolic pressure-diameter relationships obtained during phenylephrine infusion in propranolol-untreated and -treated dogs. ○ indicates normal dog; ●, 4-week-paced dog. C, Plasma norepinephrine, atrial natriuretic peptide, and angiotensin II levels before and after 4-week pacing with or without propranolol treatment.
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.

Discussion

Evidence has accumulated as to the beneficial effect of β-blockers on the prognosis of patients with heart failure. Indeed, recent long-term survival studies have proved that β-blockers decrease both mortality and morbidity in such patients.13,14 The mechanisms proposed to explain the benefits of β-blocker therapy include a reduction in sympathetic nervous activity, restoration of the β-receptor population with improved contractile performance, enhanced myocardial relaxation, and an improved cardiac efficiency with an associated reduction in heart rate.21 However, the basis of the adrenergic cascade-related myocyte abnormalities remains largely unclear.

The important new finding made in this study is that in tachycardia-induced canine heart failure, low-dose propranolol, which had only a negative chronotropic but not a negative inotropic effect in normal dogs, restored channel regulation in RyR and thereby improved cardiac function. In previous studies, β-blockers enhanced the SR Ca\(^{2+}\)-ATPase expression with increased peak of intracellular Ca\(^{2+}\) transient in human end-stage heart failure.22 and it increased the activity of SR Ca\(^{2+}\)-ATPase in a turkey model of furazolidone-induced dilated cardiomyopathy.23 In the present study, treatment with propranolol had no effect on the protein expression of SR Ca\(^{2+}\)-ATPase or on Ca\(^{2+}\) uptake function. This discrepancy might be attributable to the different experimental dose of β-blocker used, the dose used in our study being much lower than doses used in the other studies. The regulation of SR Ca\(^{2+}\)-ATPase expression might be different depending on the magnitude of the β antagonism.
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\(^{2+}\)/H\(^{1001}\) uptake function, depends on an inhibition of Ca\(^{2+}\)/H\(^{1001}\) 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\(^{24}\) reported that the \(\beta\)-selective blocker metoprolol reversed PKA-mediated hyperphosphorylation of RyR2, restored the stoichiometry of RyR2 macromolecular complex, and normalized single-channel function in a canine model of heart failure. We confirmed the beneficial effect of \(\beta\)-blockers on RyR channel function using Ca\(^{2+}\)/H\(^{1001}\) leak and site-directed fluorescent assays. This effect seems to be exerted as a class effect of \(\beta\)-blockers, because similar results were drawn regardless of the selectivity of the \(\beta\)-blocker (\(\beta\)-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,\(^{25}\) ie, progressive loss of myofilaments from cardiac myocytes\(^{26}\) or alterations in cytoskeletal proteins, as well as desensitization of \(\beta\)-adrenergic signaling.\(^{27}\) Resensitization of the \(\beta\)-adrenergic

### TABLE 2. \([\text{H}]\text{dihydro-FK506}\) and \([\text{H}]\text{ryanodine} Binding to SR Vesicles

<table>
<thead>
<tr>
<th></th>
<th>[(\text{H})]dihydro-FK506 Binding</th>
<th>[(\text{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>
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<tr>
<td>Propranolol-untreated (n=5)</td>
<td>1.49±0.30†</td>
<td>4.59±2.09*</td>
<td>1.32±0.24†</td>
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<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>
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</table>

The parameters for \([\text{H}]\text{dihydro-FK506} binding and \([\text{H}]\text{ryanodine} binding to cardiac muscle SR fractions were determined by Scatchard analysis. The ratio of the Bmax values for \([\text{H}]\text{dihydro-FK506} and \([\text{H}]\text{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 conformational changes). This may be different in other species, including human hearts. Therefore, the findings in the present study may not necessarily be converted to human hearts.

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 conformational change and prevention of Ca\textsuperscript{2+} leak from RyR), and this apparently resulted in an alteration of intracellular Ca\textsuperscript{2+} overload and a consequent prevention of the development of LV remodeling. The present results may provide a molecular basis for the common clinical observation that use of β-blockers improves prognosis among patients with heart failure.

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References
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