Correction of Defective Interdomain Interaction Within Ryanodine Receptor by Antioxidant Is a New Therapeutic Strategy Against Heart Failure

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**Background**—Defective interdomain interaction within the ryanodine receptor (RyR2) seems to play a key role in the pathogenesis of heart failure, as shown in recent studies. In the present study we investigated the effect of oxidative stress on the interdomain interaction, its outcome in the cardiac function in heart failure, and the possibility of preventing the problem with antioxidants.

**Methods and Results**—Sarcoplasmic reticulum (SR) vesicles were isolated from dog left ventricular (LV) muscle (normal or rapid ventricular pacing for 4 weeks with or without the antioxidant edaravone). In the edaravone-treated paced dogs (EV+), but not in the untreated paced dogs (EV−), normal cardiac function was restored almost completely. In the SR vesicles isolated from the EV−, oxidative stress of the RyR2 (reduction in the number of free thiols) was severe, but it was negligible in EV+. The oxidative stress of the RyR2 destabilized interdomain interactions within the RyR2 (EV−), but its effect was reversed in EV+. Abnormal Ca2+ leak through the RyR2 was found in EV− but not in EV+. The amount of the RyR2-bound FKBP12.6 was less in EV− than in normal dogs, whereas it was restored almost to a normal amount in EV+. The NO donor 3-morpholinosydnonimine (SIN-1) reproduced, in normal SR, several abnormal features seen in failing SR, such as defective interdomain interaction and abnormal Ca2+ leak. Both cell shortening and Ca2+ transients were impaired by SIN-1 in isolated normal myocytes, mimicking the pathophysiological conditions in failing myocytes. Incubation of failing myocytes with edaravone restored the normal properties.

**Conclusions**—During the development of heart failure, edaravone ameliorated the defective interdomain interaction of the RyR2. This prevented Ca2+ leak and LV remodeling, leading to an improvement of cardiac function and an attenuation of LV remodeling. (Circulation. 2005;112:3633-3643.)

**Key Words:** calcium ■ free radicals ■ heart failure ■ sarcoplasmic reticulum

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A considerable body of evidence suggests that an abnormal regulation of the intracellular Ca2+ by the sarcoplasmic reticulum (SR) is the chief pathogenic mechanism for various types of dysfunctions seen in heart failure. In our recent studies of the canine model of pacing-induced heart failure, we have demonstrated that the Ca2+-release function of the ryanodine receptor (RyR2) is defective in heart failure, presumably because of a partial loss of FKBP12.6 from the RyR2, and this defective regulation of the RyR2 causes an abnormal Ca2+ leak by mediation of a conformational change in the RyR2. Marx et al demonstrated that protein kinase A (PKA)–mediated hyperphosphorylation of the RyR2 causes dissociation of FKBP12.6 from the RyR2, resulting in a defective channel function due to an increased sensitivity to Ca2+-induced activation. In failing hearts, reduced levels of PP1 and PP2A in the RyR2 macromolecular complex rather than increased PKA activity appear to be responsible for the RyR2 hyperphosphorylation and the formation of “leaky” channels. We found that both β-blockers and a new cardioprotective agent, JTV519, correct the defective FKBP12.6-mediated control of the RyR2, improving cardiac function during the development of heart failure. Wehrens et al also demonstrated that JTV519 increased the binding affinity of FKBP12.6 to the RyR2, which stabilized the closed state of the RyR2 channels and prevented the abnormal Ca2+ leak that would otherwise have triggered ventricular arrhythmias. These studies suggest that securing the FKBP12.6-mediated stabilization of the RyR2 may be a new therapeutic strategy against heart failure.
Recently, it has been demonstrated that the mode of interdomain interaction of 2 specific domains within the RyR2 (N-terminal and central domains), which contain several mutations reported in arrhythmogenic right ventricular cardiomyopathy type 2 (ARVD/C2) and polymorphic ventricular tachycardia,7–9 plays a key role in Ca\(^{2+}\) channel regulation and in the pathogenesis of heart failure. In normal operation, the interaction of these domains is tight (zipped), stabilizing the closed state of the channel; on stimulation of the RyR2, the interdomain interaction becomes loose (unzipped), and the channel opens.10 In diseased conditions, a mutation in either of these domains weakens the interdomain interaction even in resting or nonactivating conditions, and diseased channels remain partially open. As shown in our recent study of the pacing-induced heart failure model,11 abnormal interdomain interaction as well as dissociation of the RyR2-bound FKBP12.6 destabilizes the channel gating of the RyR2 and then produces Ca\(^{2+}\) leak, suggesting that the weakened interdomain interaction is one of the key mechanisms underlying the pathogenesis of ARVD/C2, polymorphic ventricular tachycardia, and heart failure.

It has been suggested that oxygen free radicals, produced by the reduction of oxygen during many cellular reactions, are involved in the pathogenesis of a variety of cardiovascular diseases, including heart failure.12 Highly reactive free radicals have a severe effect on many cellular structures and on a variety of important cellular functions. Recently, it has been suggested that RyR channel gating is regulated by the redox state and that oxidation or nitrosylation of the cysteine residues in the RyR produces considerable changes in channel function.13 Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one; MCI-186), a synthetic antioxidant, is a ubiquitously acting direct free radical scavenger. Because of its highly efficient function of detoxifying the devastatingly reactive \(\cdot OH\), edaravone directly neutralizes peroxyl radicals (LOO\(\cdot\)).14 We hypothesized that in heart failure the production of oxygen free radicals may affect the channel regulation of the RyR2 and cause abnormal contractile and relaxation function. If this is the case, edaravone, because of its antioxidant effect, may restore normal channel gating in the failing RyR2, thereby preventing the development of heart failure.

In the present study we tested this hypothesis by investigating the effects of oxidative stress on several key factors that are likely involved in the pathogenesis, such as the interdomain interaction and the RyR2-associated FKBP12.6, as described above. As shown here, the NO donor 3-morpholinosydnonimine (SIN-1) produced, in the normal SR, the same type of dysfunctions as seen in the SR from the failing heart (pacing-induced heart failure), such as destabilization of interdomain interaction, partial dissociation of FKBP12.6, and Ca\(^{2+}\) leak. Interestingly, the antioxidant edaravone reversed all 3 of these problems in pacing-induced heart failure.

Methods

Materials
FK506, edaravone, SEA0400, and JTV519 were provided by Fuji-sawa Pharmaceutical Co Ltd, Mitsubishi Pharma Corporation Co Ltd, Taisho Pharmaceutical Co Ltd, and Aetas Pharmaceutical Co Ltd, respectively.

Animal Preparation
In beagle dogs weighing 10 to 13 kg, heart failure was induced by 28 days of rapid ventricular (RV) pacing at 250 bpm (referred to as 4W pacing) with the use of an externally programmable miniature pacemaker (Medtronic Inc), and both left ventricular (LV) pressure and 2-dimensional echocardiograms were measured in the conscious state, as described elsewhere.1,3,4 The long-term administration of the antioxidant edaravone (0.6 mg/kg per day IV) was immediately followed by the initiation of RV pacing. The care of the animals and the protocols used were in accordance with guidelines of the Animal Ethics Committee of Yamaguchi University School of Medicine.

Preparation of SR Vesicles
SR vesicles were prepared from dog LV, as described elsewhere.1,3,4

Ca\(^{2+}\) Uptake and Leak Assays
Ca\(^{2+}\) uptake and Ca\(^{2+}\) leak assays were performed as described previously.1,3,4

\[^{3}\text{H}]\text{Dihydro-FK506}\ and \[^{3}\text{H}]\text{Ryanodine Binding Assays}\n[^{3}\text{H}]\text{Dihydro-FK506}\ and \[^{3}\text{H}]\text{yanodine (Dupont NEN) binding assays were performed as described elsewhere.1,3,4}

Peptides Used and Peptide Synthesis
We used a synthetic peptide corresponding to residues 2460 to 2495 of the RyR2, Dpc10, that includes 1 residue mutable in polymorphic ventricular tachycardia, as described previously.11,15 2459GFCP-DHKAAMVLFLDHRG

The peptide was synthesized on an Applied Biosystems model 431A synthesizer with the use of Fmoc \([4-(9-fluorenyl)methoxy]carbonyl\) as the α-amino protecting group, as described previously.15

Site-Directed Fluorescence Labeling of the RyR2
Specific fluorescence labeling of the RyR2 moiety of the SR was performed with the use of the cleavable hetero-bifunctional cross-linking reagent sulfo-succinimidyl 3-(12-\(\cdot\)azido-4-methylcumarin-3-acetamido)ethyl[dithio]propionate (SAED) from PIERCE, with DPC10 as a site-specific carrier, as described previously.11,16

Fluorescence Quenching of the Methylcoumarin Acetate Probe Attached to the DPC10 Binding Site
The zipped and unzipped states of the interacting domains of the RyR2 were assessed by the fluorescence quench technique described previously.11,16 The principle of the fluorescence quench assay of domain unzipping is that a large-size quencher BSA-QSY is inaccessible to the attached methylcoumarin acetate in the zipped state, whereas it becomes accessible to the methylcoumarin acetate site in the unzipped state. To form the quencher, QSY 7 carboxylic acid was conjugated with BSA by incubating 5 mmol/L QSY 7 carboxylic acid with 0.5 mmol/L BSA in 20 mmol/L HEPES (pH 7.5) for 60 minutes at 22°C in the dark. Unreacted QSY 7 carboxylic acid was removed by means of Sephadex G50 gel filtration. Fluorescence quenching by both QSY 7 BSA conjugate (a large-size quencher) and acrylamide (a small-size quencher) was performed by measuring steady state fluorescence of labeled methylcoumarin acetate (excitation at 368 nm, emission at 455 nm) in the presence or absence of the quencher. The data were analyzed with the use of the Stern-Volmer equation.

Immunoblot Analysis
Immunoblot analyses for FKBP12.6, SR Ca\(^{2+}\)-ATPase, and phospholamban (PLB) were performed as described elsewhere.1,3,4 By employing the method of Marx et al,2 we achieved coimmunoprecipitation of FKBP12.6 from SR using anti-RyR2 antibody (Onco-gene Research Products) followed by immunoblotting with anti-
FKBP12 (C-19) antibody (Santa Cruz Biotechnology). The relative phosphorylation level of RyR was determined by immunoblotting with anti-phosphoRyR2 (P2809), which was kindly provided by Dr. Andrew R. Marks (Columbia University). Specific antibodies against phosphoserine 16-phospholamban (PLB; Upstate Biotech) and an epitope common to all PLB forms (PLB; Upstate Biotech) were also used.

**Oxidative Stress Level in LV Muscle and RyR2**

To determine the oxidative stress level in the hearts, immunohistochemical analysis was performed with the use of mouse monoclonal anti–4-hydroxy-2-nonenal (HNE)–modified protein antibody (1:50 dilution, NOF Medical Department), according to the method of Nakamura et al.17 The content of free thiols (the number of reduced cysteines) in the canine RyR2 was determined with the use of the monobromobimane (mBB, Calbiochem) fluorescence technique.18,19 SR vesicles were incubated with an excess concentration (250 μmol/L) of lipophilic, thiol-specific agent mBB for 1 hour in the dark at 24°C. The fluorescence emission intensity of the RyR2-bound mBB (ie, the thiol content) was then measured at 482 nm by exciting at 382 nm (Perkin-Elmer luminescence spectrophotometer LS50B). As a reference, matched experiments were performed in the presence of 100 μmol/L dithiothreitol (DTT).19 The mBB fluorescence in the RyR2, expressed as a percentage of the reference, was defined as the relative content of free thiols in the RyR2.

In canine cardiac myocytes, a fluorescent probe, 2′,7′-dichlorofluorescin diacetate (DCFH-DA; Molecular Probes), was used for the assessment of intracellular reactive oxygen species (ROS) formation.20 This assay is widely used as a reliable method for the measurement of intracellular ROS such as hydrogen peroxide (H2O2), hydroxyl radicals (OH·), and hydroperoxides (ROOH). Fluorescence images (excitation at 490 nm and emission at 530 nm) were acquired with a microscope (LSM 510 Carl Zeiss).

**Ca2+ Transient and Cell Shortening in Dog Myocytes**

Myocyte shortening and intracellular calcium were measured as described previously.11 In brief, myocytes were incubated with 1 μmol/L fura 2-AM, 0.0045% Pluronic F-127 (Sigma), and 0.1% dimethyl sulfoxide for 30 minutes, then washed twice with HEPES

![Figure 1. Effects of edaravone on cardiac function and neurohormonal factors.](image)

A. Representative M-mode echocardiogram. Note that both LV end-diastolic and LV end-systolic diameters were smaller in edaravone-treated dog than in edaravone-un-treated dog. B. Plasma norepinephrine, angiotensin II, and atrial natriuretic peptide (ANP) levels before and after RV pacing for 4 weeks with or without edaravone treatment. EV(−) indicates edaravone-un-treated 4-week paced group; EV(+), edaravone-treated 4-week paced group.

<table>
<thead>
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<th>TABLE 1. Hemodynamic Data</th>
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<tr>
<td>Preparing 122±9</td>
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<tr>
<td>4w pacing</td>
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<tr>
<td>Preparing 118±16</td>
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<tr>
<td>4w pacing</td>
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HR indicates heart rate; LVPSP, LV peak-systolic pressure; LVEDP, LV end-diastolic pressure; +dP/dt, peak +dP/dt of LV pressure; Tau, time constant of LV pressure decay during isovolumic relaxation period; LVEDD, LV end-diastolic diameter; LVESD, LV end-systolic diameter, and FS, fractional shortening: (LVEDD−LVESD)/LVEDD×100. Data are mean±SD.

*P<0.01 vs preparing group; †P<0.05, ‡P<0.01 vs edaravone-untreated group.
buffer containing (in mmol/L) NaCl 126, KCl 4.4, MgCl$_2$ 1.0, CaCl$_2$ 1.08, HEPES 24, glucose 11, NaOH 13, and probenecid 0.5 (pH 7.4). Cells were stimulated by a field electric stimulator (IonOptix) at 1.0 Hz. A dual-excitation spectrofluorometer was used to record fluorescence emissions (505 nm) elicited from exciting wavelengths at 340 and 380 nm. The intracellular calcium was monitored as the ratio of fluorescence of the cell at 360 and 380 nm excitation.

**Statistical Analysis**

Paired $t$ tests were performed for before and after comparisons of hemodynamic data. ANOVA was used to compare data groups. When we identified a significant trend by the F test, we used the Scheffé post hoc test to compare the data. For comparison of multiple measurements, repeated-measures analysis was used. Data are expressed as mean±SD. We accepted a probability value of <0.05 as statistically significant.

**Results**

**Determination of an Appropriate Concentration of Edaravone for Long-term Administration**

To determine appropriate concentrations of edaravone for long-term administration, we examined the concentration-dependent effect of edaravone on hemodynamic parameters in normal dogs. Various amounts of edaravone were administered intravenously for 3 days for each dose, starting at 0.1 mg/kg/day with gradual increments to 1.2 mg/kg/day. At a dose of >0.9 mg/kg/day, both LV pressure and the peak +dP/dt of LV pressure tended to decrease, while the dogs were kept in a conscious state. Therefore, we used a dose of 0.6 mg/kg/day for long-term administration of edaravone in this study to circumvent the acute hemodynamic effect of edaravone.

**Hemodynamic and Humoral Data**

In the edaravone-treated (EV+) dogs with long-term RV pacing, both systolic and diastolic functions were largely preserved, and none of these dogs developed heart failure (Table 1, Figure 1A). Plasma norepinephrine, angiotensin II, and atrial natriuretic peptide were all higher in the edaravone-untreated paced (EV−) dogs than in normal dogs (before pacing). Long-term administration of edaravone significantly reduced the levels of all of these neurohormonal factors in the paced dogs (Figure 1B). These data indicate that in the edaravone-treated dogs, there were no signs of heart failure after long-term RV pacing.

**Levels of Oxidative Stress Are Elevated in Failing Hearts**

To examine the extent of oxidative stress in the pacing-induced failing hearts, we determined the levels of HNE-modified protein in cardiac tissue. Positive immunohistochemical staining for HNE-modified protein was distinct in the failing cardiac myocytes (Figure 2A, HNE−, EV−). However, in the edaravone-treated paced hearts, positive immunohistochemical staining for HNE-modified protein was absent (Figure 2A, HNE+, EV+). The relative content of free thiols in the RyR2 (for definition, see Methods) was considerably reduced in the untreated failing hearts (EV−) but was normal in EV+ hearts (Figure 2B), indicating that the oxidation of the RyR2 is in fact involved in the oxidative stress in failing hearts. After treatment with the NO donor SIN-1, which generates the NO-related species peroxynitrite (OONO⁻), approximately half of the free thiols remained unreactive (not oxidized) in all groups, consistent with the finding seen in the RyR1.

**Effects of Edaravone on SR Ca$^{2+}$ Leak and Defective FKBP12.6-RyR2 Interaction in Failing Hearts**

Addition of 0.3 μmol/L thapsigargin to normal SR vesicles produced little Ca$^{2+}$ leak, whereas addition of 30 μmol/L FK506 together with 1 μmol/L thapsigargin produced a pronounced leak (Figure 3A). In contrast, in the failing (EV−) SR vesicles, the addition of thapsigargin alone produced a prominent Ca$^{2+}$ leak, but the addition of FK506 produced no further increase (Figure 3A). In the EV+ SR vesicles, spontaneous Ca$^{2+}$ leak was not observed, and FK506 increased Ca$^{2+}$ leak as in the normal SR (Figure 3A).
In the EV− SR vesicles, the RyR2 was PKA-hyperphosphorylated, but edaravone treatment reduced the channel phosphorylation to a level seen in the normal hearts (Figure 3B). The amount of the RyR2-associated FKBP12.6 was decreased by long-term RV pacing, but the decrease was prevented by edaravone treatment (Figure 3C).

Effects of Oxidative Stress on Ca²⁺ leak, Interdomain Interaction Within RyR2, and FKBP12.6-RyR2 Interaction

To further test the view that oxidative stress of the RyR2 is the cause of the Ca²⁺ leak, we investigated the effect of SIN-1 on the Ca²⁺ flux in normal SR. As shown in Figure 4A, 30 μmol/L SIN-1 increased the Ca²⁺ leak, and this Ca²⁺ leak was completely inhibited by 1 μmol/L JTV519, which had been found to inhibit the Ca²⁺ leak through the stabilization of the RyR2 via restoration of an interdomain interaction within the RyR2.11

To monitor the zipped and unzipped states of the interacting domains of the RyR2, we used QSY-BSA as a macromolecular quencher (Figure 4B). As in the case of our recent study with DPc10,11 the slope of the Stern-Volmer plot, which represents K₀, or the extent of domain unzipping, was considerably increased by SIN-1 (30 μmol/L), indicating that SIN-1 in fact induced a sizable opening between the interacting domains (Figure 4B, top). The SIN-1-induced increase in the extent of fluorescence quenching was almost completely reversed by 1 μmol/L JTV519 (Figure 4B, top). This is particularly important in view of our recent finding11 that JTV519 stabilizes the zipped configuration of the interacting domains and thus prevents Ca²⁺ leak. The extent of fluorescence quenching (K₀) in the EV− SR vesicles was larger than in normal SR (compare Reference 11), and the addition of SIN-1 had no appreciable effect on K₀ (Figure 4B, middle). However, JTV519 reduced the K₀ in failing SR to a normal value even in the presence of SIN-1 (Figure 4B, middle). In EV+ vesicles, K₀ values were normally restored. These results suggest that domain unzipping had already occurred in failing SR partly by oxidative stress, causing Ca²⁺ leak, and that JTV519 restored the zipped state and in turn normal channel gating.

To further resolve the mechanism by which the edaravone treatment decreased the phosphorylation level of the RyR2 and inhibited FKBP12.6 dissociation from the RyR2, we investigated the effect of oxidative stress on the amount of FKBP12.6 bound with the RyR2. As seen in Figure 4C, addition of SIN-1 had no appreciable effect on the PKA phosphorylation level of the RyR2 or on FKBP12.6 binding to the RyR2 if cAMP was absent. However, in the presence of SIN-1 (30 μmol/L), the cAMP
concentration dependence of both PKA phosphorylation of the RyR2 and FKBP12.6 dissociation was shifted toward lower concentrations of cAMP. These results suggest that domain unzipping caused by oxidative stress produces larger extents of PKA phosphorylation of the RyR2 and dissociation of FKBP12.6 from the RyR2 in the presence of cAMP at micromolar concentrations, which are presumably present in physiological conditions.

Figure 4D shows the effect of SIN-1 on the relative content of free thiols in the RyR2. SIN-1 decreased the number of free thiols of the RyR2 (i.e., oxidation). Unlike edaravone, JTV519 did not rescue the oxidative stress of the RyR2 induced by SIN-1 (data not shown), indicating that the inhibition of Ca$^{2+}$ leak by JTV519 is mediated by a mechanism independent of the RyR2 oxidation.

Effects of Edaravone on the Amount of SR Ca$^{2+}$-ATPase, Rate of Ca$^{2+}$ Uptake, and Ratio of Ser16-Phosphorylated PLB and Total PLB in Failing Hearts

After rapid RV pacing for 4 weeks, both the SR Ca$^{2+}$ uptake activity and the amount of SR Ca$^{2+}$-ATPase were reduced, and these changes were not observed in the EV+ group (Figure 5A, B). The levels of Ser16-phosphorylated PLB and total PLB among these groups of SR vesicles are compared in Figure 5C (top: gel picture; bottom: calculated values of relative phosphorylation). There was no difference in the level of total PLB among the 3 groups, but there was a significant decrease in the basal level of phosphorylated PLB in the failing EV− SR vesicles. In the EV+ SR vesicles, the level of phosphorylated PLB was restored back toward a normal level.
Effects of Edaravone or JTV 519 on Ca$^{2+}$ Transient and Cell Shortening in Normal and Failing Myocytes

We further assessed whether the adverse effects of oxidative stress on SR Ca$^{2+}$ release function and its reversal by JTV519 seen in SR vesicles can be seen in canine myocytes as well. In normal myocytes, SIN-1 decreased the peak of Ca$^{2+}$ transient and prolonged its duration in parallel with a reduced percent cell shortening (Figure 6A). Addition of either JTV519 (1 μmol/L) or edaravone (100 μmol/L) resulted in a significant, if not complete, restoration of both Ca$^{2+}$ transient and cell shortening to normal levels (Figure 6A). Several reports have suggested that ROS induces intracellular Ca$^{2+}$ overload through activation of a reversed mode of Na$^{+}$-Ca$^{2+}$ exchange during reoxygenation.22,23 Therefore, we examined the effect of the specific inhibitor of Na$^{+}$-Ca$^{2+}$ exchange, SEA0400, on normal myocyte function. SEA0400 (1 μmol/L) had no appreciable effect on cell shortening and peak Ca$^{2+}$ transient at baseline. In the presence of SEA0400, SIN-1 decreased the peak of Ca$^{2+}$ transient and prolonged its duration in parallel with a reduced percent cell shortening to an extent similar to those in the absence of SEA0400 (Figure 6A). This finding excludes the possibility that the Na$^{+}$-Ca$^{2+}$ exchange may have been involved in the observed effects of SIN-1 on normal cardiomyocytes, although other radicals may influence calcium influx through reverse-mode Na$^{+}$-Ca$^{2+}$ exchange activity. As shown in Figure 6B, Ca$^{2+}$ transient and cell shortening in the myocytes isolated from pacing-induced failing dog hearts were deteriorated compared with those in normal myocytes. In the presence of isoproterenol, both Ca$^{2+}$ transient and cell shortening were partially restored toward normal by incubation with edaravone (100 μmol/L) for 12 hours and also with JTV519 (1 μmol/L), which reverses domain unzipping.11 Table 2 summarizes the cell shortening and Ca$^{2+}$ transient data in normal and failing myocytes. Thus, all the data obtained from the experiments with SR vesicles and myocytes consistently support the notion that oxidative stress–induced cardiac dysfunction is induced by defective interdomain interaction in the RyR2 and that its reversal either directly by JTV519 or indirectly by edaravone restores normal cardiac function.

Figure 6C shows fluorescence images after application of the fluorescent probe of intracellular ROS, DCFH-DA (1 μmol/L), into the normal and failing cardiomyocytes. In normal cardiomyocytes, fluorescence intensity was markedly increased after addition of 100 μmol/L H$_2$O$_2$ or 30 μmol/L SIN-1, whereas it was restored to a normal level in the presence of 100 μmol/L edaravone. In failing cardiomyo-
cytes, fluorescence intensity had already been increased to a high level, but a normal level of fluorescence intensity was restored by application of 100 μmol/L edaravone (Figure 6C) but not by 1 μmol/L JTV519 (data not shown). These findings indicate that the ROS level is indeed increased in failing myocytes, and it can be reversed by the antioxidant edaravone.

Discussion

Many reports suggested that oxidative stress caused by oxygen free radicals is in fact involved in the pathogenesis of heart failure. Several reports have shown that there is an increased oxygen-derived free radical production in heart failure, and it can be reversed by β-blocker therapy. Recent reports also showed that the RyR is the target of ROS and that the channel activity of the RyR is regulated by oxidation or nitrosylation. S-Nitrosylation of the RyR2 may have a physiological significance in the normal heart, as suggested by several reports. However, oxidative stress often results in a deleterious loss of normal cardiac function, leading to heart failure.

The most important aspect of this study is the finding that the pacing-induced heart failure and the oxidative stress–induced contractile dysfunction share at least some common causative molecular mechanisms, namely, defective interdomain interaction accompanied by a decrease in the amount of the RyR2-bound FKBP12.6 and an increase in the extent of phosphorylation. This conclusion is supported by several findings made in the present study. First, administration of the antioxidant edaravone during the course of RV pacing prevented the development of heart failure, as shown consistently in the hemodynamic, hormonal, and immunohistochemical data. The treatment of the SR isolated from normal heart with the oxidant SIN-1 resulted in a considerable reduction in the content of reactive thiols in the RyR2 moiety. In the SR from failing hearts subjected to the RV pacing, the content of reactive thiols was considerably reduced, indicating that oxidative stress has occurred within the RyR2; however, the thiol content in the SR from the edaravone-treated hearts was essentially identical with that of the normal control even after RV pacing for 4 weeks. The SIN-1–induced oxidative
stress within the RyR2 of normal SR resulted in an increased extent of domain unzipping (defective interdomain interaction). Likewise, the RV pacing resulted in the defective interdomain interaction even without the treatment with SIN-1, but edaravone administration during the pacing prevented the defective interdomain interaction from occurring. SR Ca\(^{2+}\) leak was increased by SIN-1–mediated oxidation of normal SR or RV pacing. The SR Ca\(^{2+}\) leak produced by RV pacing was prevented by administration of edaravone during the pacing. Finally, these observations with the isolated SR (SR Ca\(^{2+}\) leak induced by RV pacing or oxidative stress) were reproduced in similar abnormal features of cell shortening and Ca\(^{2+}\) transients of the cardiac myocytes.

The next important question concerns the molecular mechanism underlying the pathogenesis of the in vivo pacing-induced heart failure and the oxidative stress–induced contractile dysfunction in myocytes. As shown in this study, RV pacing as well as oxidation of the RyR2 with SIN-1 weakened the interdomain interaction (ie, domain unzipping). Importantly, the RV pacing–induced problem in the interdomain interaction was corrected not only by JTV519, which also corrected SR Ca\(^{2+}\) leak in the RV-paced heart, but also by edaravone, which corrected the leak problem in the heart subjected to oxidative stress. Similarly, the SIN-1–induced problem in the interdomain interaction was corrected not only by the antioxidant edaravone but also by JTV519. This clearly indicates that the defect in the mode of interdomain interaction is the source mechanism of pathogenesis of both types (ie, RV pacing and oxidation) of failing hearts.

According to Baker et al,\(^{29}\) the RyR1 has an oxidoreductase-like domain in the N-terminal region 41 to 420, which may function as a redox sensor. This region corresponds to the N-terminal domain, one of the key domains involved in the interdomain interaction. Voss et al\(^{30}\) identified several thios of the RyR1 that are highly reactive to the thiol-directed fluorescence reagent 7-diethylamino-3-(4’-maleimidophenyl)-4-methylcoumarin (hyperreactive thiol[s]). Interestingly, more than half of the corresponding Cys residues in the RyR2 (2403, 2532, 2573, 2578) are clustered in the region that is partially overlapping with the central domain, another key domain involved in the interdomain interaction. It seems very likely that the interdomain interaction is under the control of redox-sensing mechanisms built into these domains. There are a number of reports of oxidation-induced activation of the RyR Ca\(^{2+}\) channels with a variety of oxidative reagents.\(^{13,19,20,31}\) The present data suggest that the oxidation-induced domain unzipping might be a basic mechanism for the widely observed oxidation-induced SR Ca\(^{2+}\) release. Apart from oxidation, specific nitrosylation of Cys-3635, which was found to modify RyR1 channel activity,\(^{32}\) may also be involved in the defective RyR2 channel gating in heart failure.

In the dog model of heart failure produced by 4W pacing, the defective interdomain interaction is accompanied by 2 other major problems: reduction in the amount of the RyR2-bound FKBP12.6 and an increase in the level of cAMP-dependent phosphorylation of the RyR2.\(^{11}\) It is rather difficult to delineate the sequence of the occurrence of these events because these 3 problems appear to occur in a coordinated manner. The fact that, in the presence of cAMP, SIN-1 increased PKA phosphorylation and FKBP12.6 dissociation suggests that phosphorylation and FKBP12.6 dissociation accelerate the SIN-1–induced domain unzipping, and domain unzipping accelerates phosphorylation and FKBP12.6 disso-

### TABLE 2. In Vivo Cell Shortening and Ca\(^{2+}\) Transient in Normal and Failing Myocytes

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<th>Untreated (n=30)</th>
<th>+JTV5519 (n=30)</th>
<th>+Edaravone (n=30)</th>
<th>+SEA0400 (n=10)</th>
<th>EV− (n=30)</th>
<th>EV+ (n=30)</th>
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<td>Cell shortening, % decrease from baseline</td>
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<td>2.0±0.8§</td>
<td>4.7±1.1†</td>
</tr>
<tr>
<td>SIN-1 30 μmol/L</td>
<td>5.2±1.8*</td>
<td>8.2±2.5‡</td>
<td>8.7±1.9‡</td>
<td>4.4±0.7*</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>SIN-1 100 μmol/L</td>
<td>2.1±0.9†</td>
<td>6.4±2.2§</td>
<td>6.9±2.6§</td>
<td>2.6±0.6†</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Isoproterenol 50 nmol/L</td>
<td>14.8±4.5†</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>2.5±1.1§</td>
<td>8.9±2.6†</td>
</tr>
<tr>
<td>Peak of Ca(^{2+}) transient, % increase from baseline</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SIN-1−</td>
<td>33.0±3.4</td>
<td>31.3±4.0</td>
<td>33.2±4.1</td>
<td>31.6±2.6</td>
<td>16.7±4.3‡</td>
<td>25.9±4.6‡</td>
</tr>
<tr>
<td>SIN-1 30 μmol/L</td>
<td>26.5±3.1*</td>
<td>28.7±3.8</td>
<td>32.7±4.3</td>
<td>27.6±1.9*</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>SIN-1 100 μmol/L</td>
<td>14.4±3.7†</td>
<td>25.8±3.1‡</td>
<td>27.8±3.9‡</td>
<td>14.6±1.7†</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Isoproterenol 50 nmol/L</td>
<td>58.4±15.8†</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>22.8±2.5§</td>
<td>39.7±14.1†</td>
</tr>
<tr>
<td>Time from peak to 70% decline, ms</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SIN-1−</td>
<td>90.6±10.6</td>
<td>90.0±22.4</td>
<td>88.9±7.7</td>
<td>92.1±14.5</td>
<td>118.6±13.5†</td>
<td>98.6±8.3</td>
</tr>
<tr>
<td>SIN-1 30 μmol/L</td>
<td>109.1±2.1*</td>
<td>97.1±14.5</td>
<td>92.1±6.8</td>
<td>112.0±5.6*</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>SIN-1 100 μmol/L</td>
<td>115.3±6.4†</td>
<td>108.0±6.4*</td>
<td>93.7±10.3‡</td>
<td>120.6±4.5†</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Isoproterenol 50 nmol/L</td>
<td>56.9±5.7†</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>110.7±15.8§</td>
<td>58.2±8.9†</td>
</tr>
</tbody>
</table>

*P<0.05, †P<0.01 vs SIN-1−; §P<0.05, ‡P<0.01 vs untreated normal myocytes.
cation, letting these 3 events occur in a concerted manner. Thus, like JTV519, the antioxidant edaravone corrected all of these problems in a concerted manner.

In addition to the effect of edaravone on the mode of interdomain interaction, we should consider various other pharmacological functions of this drug. Long-term administration of edaravone improved the Ca²⁺ uptake function, SERCA2a expression, and PLB phosphorylation. These effects appear to be similar to that of β-blockers or the angiotensin II receptor antagonist valsartan, both of which reduce sympathetic activity and thus prevent Ca²⁺ leak.³⁻³³ However, because edaravone does not have a β-blocking effect by itself, the beneficial effect of edaravone on Ca²⁺ uptake may be relevant to the inhibitory effect on intracellular Ca²⁺ overload. The inhibition of LV dilatation by edaravone may be attributable to other possible mechanisms by which antioxidants prevent the progression of heart failure. Because improvement of LV function after administration of edaravone may decrease the sympathetic activity as a secondary effect, it would be rather difficult to evaluate the specific effect of edaravone as distinct from the catecholamine effect.

In conclusion, administration of the antioxidant edaravone during RV pacing of the canine heart prevented the development of heart failure by correcting several problems occurring in the RyR2 moiety of the SR of failing hearts, such as defective interaction of the regulatory domains in the RyR2, partial dissociation of RKB12.6, PKA hyperphosphorylation, and Ca²⁺ leak. Oxidation of the cysteine residues of the RyR2 with SIN-1 destabilized the interdomain interaction, mimicking the situation in the failing SR, but on treatment of the SR with edaravone the normal mode of interdomain interaction was restored. The present study suggests that the use of free radical scavengers will be a promising therapeutic strategy against heart failure.

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Disclosures

The authors report no conflicts of interest.

References


Correction of Defective Interdomain Interaction Within Ryanodine Receptor by Antioxidant Is a New Therapeutic Strategy Against Heart Failure

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