Reversal of Global Apoptosis and Regional Stress Kinase Activation by Cardiac Resynchronization

Khalid Chakir, PhD; Samantapudi K. Daya, MD; Richard S. Tunin, BS; Robert H. Helm, MD; Melissa J. Byrne, PhD; Veronica L. Dimaano, MD; Albert C. Lardo, PhD; Theodore P. Abraham, MD; Gordon F. Tomaselli, MD; David A. Kass, MD

Background—Cardiac dyssynchrony in the failing heart worsens global function and efficiency and generates regional loading disparities that may exacerbate stress-response molecular signaling and worsen cell survival. We hypothesized that cardiac resynchronization (CRT) from biventricular stimulation reverses such molecular abnormalities at the regional and global levels.

Methods and Results—Adult dogs (n = 27) underwent left bundle-branch radiofrequency ablation, prolonging the QRS by 100%. Dogs were first subjected to 3 weeks of atrial tachypacing (200 bpm) to induce dyssynchronous heart failure (DHF) and then randomized to either 3 weeks of additional atrial tachypacing (DHF) or biventricular tachypacing (CRT). At 6 weeks, ejection fraction improved in CRT (2.8 ± 1.8%) compared with DHF (-4.4 ± 2.7; P = 0.02 versus CRT) dogs, although both groups remained in failure with similarly elevated diastolic pressures and reduced dP/dtmax. In DHF, mitogen-activated kinase p38 and calcium-calmodulin–dependent kinase were disproportionally expressed/activated (50% to 150%), and tumor necrosis factor-α increased in the late-contracting (higher-stress) lateral versus septal wall. These disparities were absent with CRT. Apoptosis assessed by terminal deoxynucleotide transferase-mediated dUTP nick-end labeling staining, caspase-3 activity, and nuclear poly ADP-ribose polymerase cleavage was less in CRT than DHF hearts and was accompanied by increased Akt phosphorylation/activity. Bcl-2 and BAD protein diminished with DHF but were restored by CRT, accompanied by marked BAD phosphorylation, enhanced BAD–14-3-3 interaction, and reduced phosphatase PP1, consistent with antiapoptotic effects. Other Akt-coupled modulators of apoptosis (FOXO-3 and GSK3β) were more phosphorylated in DHF than CRT and thus less involved.

Conclusions—CRT reverses regional and global molecular remodeling, generating more homogeneous activation of stress kinases and reducing apoptosis. Such changes are important benefits from CRT that likely improve cardiac performance and outcome. (Circulation. 2008;117:1369-1377.)

Key Words: cardiac pacing, artificial ■ apoptosis ■ heart failure ■ molecular biology ■ pacing

Chronic heart failure involves a progressive decline in cardiac performance accompanied by chamber dilation and multiple abnormalities of molecular signaling that adversely affect cell function and survival.1–3 These processes and clinical outcomes are further worsened if hearts develop dyssynchronous contraction resulting from an intraventricular conduction delay.4–6 Mechanical dyssynchrony generates marked disparities in regional loading7,8 and reduces systolic function at similar or higher metabolic demand compared with synchronous failing hearts.8 The early activated region is less loaded because its shortening reciprocally stretches the still inactive opposing wall, whereas the late-stimulated region must operate at higher loads because of prestretch and late contraction against already stiffened muscle.10,11 Such unequal loading can affect localized expression/activity of calcium handling, stress kinases, and electric conduction proteins in the high-load lateral wall.12 Dyssynchrony also triggers global changes by adversely affecting chamber mechanoenergetics.13

Clinical Perspective p 1377

Biventricular stimulation, now called cardiac resynchronization therapy (CRT), offsets underlying conduction delay to homogenize loading and shortening. Its development as a clinical therapy marked an important advance for heart failure, and it remains arguably the only treatment to date that enhances systolic function while improving long-term outcome and survival.14–16 Studies exploring the mechanisms for CRT have focused primarily on chamber mechanics and energetics,13,17–21 with virtually all the work being performed in patients. No prior studies have tested whether and how CRT affects regional...
and/or global molecular protein signaling abnormalities induced by dyssynchronous heart failure (DHF).

The present study tested the hypothesis that CRT reverses chronic regional and global molecular stress cell-survival signaling abnormalities in failing hearts with electromechanical dysynchrony. To this end, we developed a novel canine model in which animals first undergo radiofrequency ablation of the left bundle branch and are then subjected to either 6 weeks of atrial tachypacing (DHF) or 3 weeks of such pacing followed by 3 weeks of biventricular tachypacing. Both models involve tachypacing for 6 weeks, with the primary difference being the synchrony of contraction during the latter half-period, allowing a more focused analysis of the impact of CRT. We report that CRT restores normal homogeneity of stress proteins, reducing their differential expression and activation in the late-contracting lateral wall of dyssynchronous hearts. Most strikingly, we reveal a potent effect of CRT on globally reducing apoptosis via the Akt-BAD signaling pathway.

Methods

Experimental Model

Adult mongrel dogs (n=22) underwent left bundle-branch radiofrequency ablation as previously described.12,22 Left bundle-branch block was confirmed by intracardiac electrograms, with surface QRS widening from 50±7 to 104±7 ms (P<0.001). Eleven animals were paced from the right atrium for 6 weeks at ~200 bpm (AAO pacing, DHF); the remaining dogs were subjected first to 3 weeks of atrial pacing (dyssynchrony) followed by 3 weeks of biventricular tachypacing (VOO) at the same rate (CRT). Biventricular pacing was achieved by simultaneous lateral epicardial and right ventricular anterolateral free wall stimulation. Echo and tissue Doppler studies were performed at 3 and 6 weeks in conscious animals to assess left ventricular (LV) dyssynchrony, chamber dimensions, and ejection fraction. Tachypacing was suspended for 1 hour before data acquisition, and then DHF or CRT was restarted at 30 bpm above sinus rhythm (~190 bpm) to measure function at a similar rate among the animals. At the end of the study, animals were anesthetized with pentobarbital, pacing was suspended, and invasive LV pressures were recorded. Hearts were then extracted under cold cardioplegia, dissected into endocardial and mid/epicardial segments from the septum (ie, LV and right ventricular septum) and LV lateral wall, and frozen in liquid nitrogen or placed in 10% formalin.

In Vivo Cardiac Function Assessments

Transthoracic echocardiography and tissue Doppler examination were performed (GE Vivid-7, 3.5-MHz multifrequency broadband transducer, GE Healthcare, Piscataway, NJ) to measure end-diastolic and end-systolic volumes, ejection fraction, and dyssynchrony index (SD of radial strains from 12 different regions). In a subgroup of animals (n=5 in each group), dyssynchrony also was measured from magnetic resonance–tagged images (GE Signa, 1.5 T, GE Healthcare) by the circumferential uniformity ration estimate.11

To assess short-term cardiac effects of biventricular pacing in our model of DHF, a separate group of 5 animals were instrumented long term for pressure-volume analysis as previously described.23 Pressure-volume relations were measured after 3 weeks of dyssynchronous tachypacing, and atrial versus short-term biventricular stimulation were compared. Pacing was first suspended for at least 1 hour; then, heart rate was maintained constant at just above the sinus rate for the comparisons. Load-independent measures of systolic function were obtained.23 All of the animal protocols and procedures were approved by the Animal Care and Use Committee of Johns Hopkins University.

Protein Expression and Activity Analysis

Myocardial tissue was homogenized in lysis buffer (Cell Signaling Technology, Danvers, Mass), and gel electrophoresis was performed with 50 to 100 μg total protein per lane. Proteins were transferred to nitrocellulose membranes that were blocked, probed overnight at 4°C with primary antibodies, and then exposed to horseradish peroxidase–mediated dUTP nick-end labeling (TUNEL) in formalin-fixed myocardial samples (CardioTACS In Situ Apoptosis Detection Kit, International) following the manufacturer’s instructions.

Analysis of Apoptosis

Ventricular myocardial apoptosis was assessed using several complementary methods. First, we assessed terminal deoxynucleotid transferase–mediated dUTP nick-end labeling (TUNEL) in formalin-fixed myocardial samples (CardioTACS In Situ Apoptosis Detection Kit, International) following the manufacturer’s instructions.
TA5353) according to the manufacturer’s instructions. Apoptotic myocytes were counted under ×400 magnification and expressed as a fraction of cells per field. Second, apoptosis was assessed by caspase-3 proteolytic activity with a spectrophotometric assay (Fiena kit, Roche Applied Science, Indianapolis, Ind) normalized to total lysate protein and incubation time (FU · mg⁻¹ · h⁻¹). Third, we determined PARP cleavage as a marker of activated apoptotic cascades.

### Statistical Analysis

Comparisons between groups (unpaired samples) were performed by 1-way ANOVA with Bonferroni correction; comparisons between regions or myocardial layers or over time (ie, paired samples) were performed by repeated-measures ANOVA. Data are presented as mean±SEM.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

## Results

### Mechanical Dyssynchrony and Heart Failure in DHF and CRT Hearts

Figure 1A shows representative anterior and lateral strain tracings from dogs with a left bundle-branch block after 3 weeks of atrial tachypacing. Early septal shortening with concomitant lateral stretch was followed by the opposite patterns in later systole. In DHF dogs, dyssynchrony persisted for the ensuing 3 weeks of tachypacing, whereas it declined markedly in dogs receiving CRT during this period (Figure 1A and 1B). Similar differences were confirmed by magnetic

<table>
<thead>
<tr>
<th>Group</th>
<th>Td, ms</th>
<th>LVEDV, mL</th>
<th>LVESV, mL</th>
<th>LVEF, %</th>
<th>LVSV, mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>30.65±2.3†</td>
<td>51.7±2.8*</td>
<td>17.1±1.9*</td>
<td>66.7±3.1*</td>
<td>34.6±2.7</td>
</tr>
<tr>
<td>DHF</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 3 (AOO)</td>
<td>71.1±6.5</td>
<td>75.5±4.5</td>
<td>52.8±4.5</td>
<td>30.1±3.5</td>
<td>22.6±3.1</td>
</tr>
<tr>
<td>Week 6 (AOO)</td>
<td>74.2±7.6</td>
<td>87.8±5.7</td>
<td>64.6±5.2</td>
<td>25.6±3.7</td>
<td>23.2±3.6</td>
</tr>
<tr>
<td>CRT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 3 (AOO)</td>
<td>63.9±7.3</td>
<td>83.1±4.6</td>
<td>59.6±4.1</td>
<td>28.1±3.1</td>
<td>23.4±3.1</td>
</tr>
<tr>
<td>Week 6 (BV-V00)</td>
<td>29.7±7.0‡</td>
<td>93.3±6.1</td>
<td>63.8±4.5</td>
<td>30.9±3.3</td>
<td>29.4±4.5</td>
</tr>
<tr>
<td>P</td>
<td>0.004</td>
<td>0.8</td>
<td>0.02</td>
<td>0.06</td>
<td></td>
</tr>
</tbody>
</table>

Td indicates cardiac dyssynchrony; LVEDV, LV end-diastolic volume; LVESV, LV end-systolic volume; LVEF, LV ejection fraction; LVSV, LV stroke volume; and Bi, biventricular. Results after 3 weeks of AOO pacing (left bundle-branch block; DHF) are shown for each group; paired results after 3 additional weeks of either AOO pacing (DHF group) or biventricular V00 pacing (CRT group) also are provided. P values reflect a Kruskal-Wallis test of the paired differences between data at weeks 3 and 6 compared between the 2 groups. *P<0.005 vs 3- and 6-week time points in both DHF and CRT groups; †P<0.05 vs control and ‡P<0.05 vs DHF group at 3-week time point. P<0.01 vs DHF and P<0.001 (1.0) vs CRT at 6-week time point. ¶P<0.001 vs DHF at 6-weeks.

#### Table 1. Echocardiographic Assessment of Cardiac Dyssynchrony and LV Volumes, Ejection Fraction, and Stroke Volume in both DHF and CRT Animals

#### Table 2. Short-Term Improvement in Cardiac Systolic Function Induced by Biventricular Pacing in Dogs With Left Bundle-Branch Block and Heart Failure Induced by 3 Weeks of AOO Tachypacing

<table>
<thead>
<tr>
<th></th>
<th>Dysynchronous (AOO)</th>
<th>Biventricular Pacing (V00)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak systolic pressure, mm Hg</td>
<td>98.0±4.9</td>
<td>101.8±5.9</td>
<td>&gt;0.2</td>
</tr>
<tr>
<td>LV end-diastolic pressure, mm Hg</td>
<td>35.3±3.1</td>
<td>31.4±4.4</td>
<td>&gt;0.2</td>
</tr>
<tr>
<td>dP/dtmax/EDV⁻¹, mm Hg · s⁻¹ · mL⁻¹</td>
<td>10.6±1.1</td>
<td>13.5±1.5</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>End-systolic elastance, mm Hg/mL</td>
<td>5.07±0.84</td>
<td>5.7±1.0</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>PRSW, mm Hg</td>
<td>44.6±5.0</td>
<td>57.8±1.6</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>τ (relaxation), ms</td>
<td>19.1±2.9</td>
<td>18.0±1.5</td>
<td>&gt;0.2</td>
</tr>
</tbody>
</table>

EDV indicates end-diastolic volume; PRSW, preload recruitable stroke work. These studies were conducted in a separate set of long-term–instrumented dogs. P is for paired t-test comparing the 2 pacing conditions.
resonance imaging (circumferential uniformity ratio estimate index: 1=synchronous, 0=maximal dyssynchrony; normal controls, 0.97±0.01; CRT, 0.87±0.06; DHF, 0.58±0.09; P<0.01 versus other groups).

Both groups developed similar LV dysfunction after 3 weeks of pacing (ie, before randomization; Table 1), whereas by 6 weeks, ejection declined further in DHF (4.7±2.7%) but rose slightly in CRT animals (2.8±1.8%; P=0.02 versus DHF; Figure 1B), associated with a borderline higher stroke volume (P=0.058). Despite these changes, both groups had substantial persistent heart failure with similarly elevated end-diastolic pressures (36±9.7 and 33.5±3.2 mm Hg, DHF versus CRT, respectively; 9.1±2.0 mm Hg, reference control) and reduced dP/dtmax (1031±86.2 and 1062±80 mm Hg/s, DHF versus CRT, respectively; 2891.3±39 mm Hg/s, controls).

To confirm that biventricular stimulation (CRT mode) produced a short-term improvement in systolic function as in clinical studies,4 a separate group of long-term–instrumented animals with DHF were studied. Biventricular stimulation led to short-term improvement in load-independent measures of systolic function on the basis of pressure–volume analysis (Table 2).

Regional Heterogeneity of Stress Kinases With DHF Is Homogenized by CRT

DHF hearts had locally enhanced protein expression and activation of multiple stress-response proteins. For example, p38 MAP kinase (MAPK) and CaMKII increased in the late-contracting lateral wall compared with septum of DHF hearts (both endocardial and epicardial layers), but this was reduced and rendered more homogeneous by CRT (Figure 2A). These results closely correlated with enzyme activity (Figure 2B). Regional expression disparities also were observed for JNK and ERK1/2 MAPK in DHF hearts, and again these were homogenized by CRT (Figure I of the online-only Data Supplement). Regional stimulation disparities also were observed in TNF-α in DHF but not CRT hearts (Figure 2C). We previously showed that disproportionate regional activation is not observed in synchronous heart failure12,24 or in normal controls (online-only Data Supplement Figure II).

DHF Myocardium Has Increased Apoptosis That Is Reduced by CRT

One potential consequence of p38, JNK, CaMKII, and TNF-α activation is increased apoptosis.5 Figure 3A shows TUNEL staining in myocardium from control, DHF, and CRT hearts;
binding to the Bcl-x(L) complex triggers apoptosis, but on assessing their potential role in the decline in apoptosis, BAD and CRT ventricles, we examined these downstream cascades to modulators, notably BAD, GSK3

Akt regulates apoptosis by phosphorylating several negative BAD Phosphorylation

Akt Kinase Is Activated by CRT

Unlike the regional amplification of stress kinases, apoptosis changes were more global in nature, leading us to speculate that alternative pathways might be involved. One prominent cell-survival pathway is linked to activated (phosphorylated) Akt (PKB) kinase. pAkt was markedly and globally reduced in DHF vs CRT hearts (Figure 4A) yet rose to control levels in both myocardial layers (Figure 4A) yet rose to control levels with CRT. Interpretation of this assay, however, also was influenced by the changes in both total proteins.

BAD expression changes were paralleled by Bcl-2 (Figure 6A), which also binds to BAD, suggesting coordinated modification. Intriguingly, mRNA expression assessed by quantitative polymerase chain reaction was unaltered for either protein (data not shown), indicating that this regulation occurred at the translational and/or posttranslational level. We further examined BAD and Bcl-2 levels in isolated mitochondrial fractions (Figure 6B) because increases there would be considered proapoptotic. With DHF, mitochondrial BAD and Bcl-2 increased but declined with CRT. Mitochondrial cytochrome c oxidase-IV served as a loading control.

BAD also can be phosphorylated at S112 to confer antiapoptotic effects. Phosphorylation at this site was depressed in positive nuclei increased 3- to 4-fold in DHF versus control hearts but declined with CRT. Proapoptotic caspase-3 activity was greater in DHF than CRT myocardium (Figure 3B), accompanied by an increase in PARP cleavage (Figure 3C). PARP is a 116-kDa protein involved in DNA repair that is cleaved by caspase-3 to yield 89- and 24-kDa fragments. DHF hearts displayed substantial PARP fragmentation, whereas CRT ventricles displayed less PARP fragmentation. DHF hearts displayed substantial PARP cleavage (Figure 3C), was greater in DHF than CRT myocardium (Figure 3B), but returned to control levels with CRT (Figure 5A). Total BAD was markedly and globally reduced (in both myocardial layers) but returned to control levels with CRT (Figure 5A). Total BAD also declined with DHF (Figure 5B) but was similarly reduced in CRT hearts, so the disparity in pBAD could not be attributed to differences in total protein per se.

We next examined 14-3-3 protein, which binds to pBAD to suppress apoptosis. As shown in Figure 5C, full-length 14-3-3 declined in both DHF and CRT conditions, much like total BAD. However, 14-3-3 also underwent cleavage in DHF hearts, a phenomenon linked to caspase-3 activation and thought to be proapoptotic by reducing pBAD–14-3-3 interaction. The 14-3-3 cleavage declined in CRT hearts. Finally, we performed immunoprecipitation to BAD and then probed for 14-3-3 binding (Figure 5D), which declined in DHF hearts but rose again with CRT. Interpretation of this assay, however, also was influenced by the changes in both total proteins.

CRT Modifies BAD/Bcl-2 Expression and BAD Phosphorylation

Akt regulates apoptosis by phosphorylating several negative modulators, notably BAD, GSK3β, and forkhead proteins (eg, FOXO3α). Given the marked disparity of Akt activity in DHF and CRT ventricles, we examined these downstream cascades to assess their potential role in the decline in apoptosis. BAD binding to the Bcl-x(L) complex triggers apoptosis, but on phosphorylation, BAD dissociates and binds to 14-3-3 protein, suppressing apoptosis. In DHF hearts, S136-pBAD (Akt site) was markedly and globally reduced (in both myocardial layers) but returned to control levels with CRT (Figure 5A). Total BAD also declined with DHF (Figure 5B) but was similarly reduced in CRT hearts, so the disparity in pBAD could not be attributed to differences in total protein per se.

We next examined 14-3-3 protein, which binds to pBAD to suppress apoptosis. As shown in Figure 5C, full-length 14-3-3 declined in both DHF and CRT conditions, much like total BAD. However, 14-3-3 also underwent cleavage in DHF hearts, a phenomenon linked to caspase-3 activation and thought to be proapoptotic by reducing pBAD–14-3-3 interaction. The 14-3-3 cleavage declined in CRT hearts. Finally, we performed immunoprecipitation to BAD and then probed for 14-3-3 binding (Figure 5D), which declined in DHF hearts but rose again with CRT. Interpretation of this assay, however, also was influenced by the changes in both total proteins.

BAD expression changes were paralleled by Bcl-2 (Figure 6A), which also binds to BAD, suggesting coordinated modification. Intriguingly, mRNA expression assessed by quantitative polymerase chain reaction was unaltered for either protein (data not shown), indicating that this regulation occurred at the translational and/or posttranslational level. We further examined BAD and Bcl-2 levels in isolated mitochondrial fractions (Figure 6B) because increases there would be considered proapoptotic. With DHF, mitochondrial BAD and Bcl-2 increased but declined with CRT. Mitochondrial cytochrome c oxidase-IV served as a loading control.
DHF hearts and recovered toward control with CRT (Figure 7A). Rather than Akt, BAD S112 phosphorylation is induced by p90RSK, which in turn is stimulated by MAPKs and protein kinase C. Therefore, we tested whether p90RSK was differentially activated in CRT hearts. However, p90RSK phosphorylation was actually higher in DHF than CRT hearts (Figure 7B; changes in total protein followed a similar pattern [data not shown]), making it unlikely to explain altered S112-pBAD. An alternative mechanism could be reduced S112 dephosphorylation by phosphatase PP1α in CRT, and this was observed (Figure 7C). PP1β and PP1γ were unaltered by either condition (data not shown).

Finally, we assess 2 other proteins that can negatively modulate apoptosis when phosphorylated by Akt: FOXO3α and GSK3β. Unlike BAD, however, these proteins were more phosphorylated (inactivated) in DHF hearts, whereas they returned to normal levels with CRT (Figure 8). This finding is opposite to what we observed with Akt or BAD, suggesting that the latter dominated.

**Discussion**

Cardiac resynchronization directly alters the timing of electric activation with the goal of rendering contraction more homogeneous. This affects regional loading and shortening and chamber function and efficiency. To date, the consequences of CRT have been revealed almost entirely at the chamber level, with studies showing its capacity to suppress progressive dilation. Here, we show that CRT also potently influences regional and global molecular abnormalities in dyssynchronous failing hearts, lowering regional activation of stress-response kinases and globally enhancing cell-survival signaling. As all of these changes occurred despite persistent albeit slightly mitigated heart failure, they may reflect primary factors contributing to CRT benefits.

In a prior study, we first reported that DHF induces localized changes in activity and/or expression of stress-response kinases, calcium handling, and gap junction proteins in the late-activated endocardium. This was not observed in synchronous heart failure, normal hearts, or nonfailing dys-synchronous hearts, the last case highlighting the importance of combining heart failure pathophysiology with discordant contraction. The present study extends these observations, confirming amplified stress kinase activation mostly in the late-contracting lateral wall; however, the exact proteins involved differed somewhat from those in our earlier study (eg, p38 was not activated previously), and the changes were transmural. The only real difference between models was the duration of tachypacing, which was doubled for the present study, and this could underlie the disparities.

The present results are the first to show that CRT homogenizes local stress kinase expression and activity, and this is potentially...
important given the impact of these proteins on muscle function, survival, and fibrosis. p38 MAPK stimulates fibrosis and apoptosis, and is associated with contractile failure. CaMKII is survival, and fibrosis. p38 MAPK stimulates fibrosis and apoptosis, and cardiac hypertrophy coupled to histone deacetylase, and cardiac ptosis and is associated with contractile failure. CaMKII is survival, and fibrosis. p38 MAPK stimulates fibrosis and apoptosis, and cardiac hypertrophy coupled to histone deacetylase.

Figure 6. A, Protein expression (total cell lysates) of Bcl-2 declines in DHF but is restored to control levels by CRT (*P<0.05 vs control [Con] and CRT). B, Mitochondrial (Mito) fraction BAD and Bcl-2 protein expression increases with DHF and declines to control with CRT (*P<0.05 vs control and CRT). Cytochrome c oxidase-IV (COX-IV) is shown as loading control.

Figure 7. A, Phosphorylation of BAD at S112 declined with DHF but was restored toward control by CRT (*P<0.05 vs control [Con] and CRT). B, Phosphorylated p90RSK (2 different anti-sera recognizing different phosphorylation sites) shows increases with DHF but not CRT hearts (GAPDH is shown as loading control), opposite to that expected if p90RSK activity modulated S112-BAD phosphorylation (*P<0.001 vs control and CRT; †P<0.05 vs control and CRT). C, Phosphatase PP1α expression declined in CRT hearts (*P<0.01 vs control and CRT).

Figure 8. Phosphorylation of GSK3β and FOXO3α, both Akt-targeted proteins involved with cell-survival signaling, was increased in DHF but not CRT hearts. Akt S136 phosphorylation is again shown by contrast, and GAPDH is provided for loading control. Summary densitometry results are shown on the right. *P<0.05 vs control and CRT.

triggered by abnormal loading, and its decline solely in the lateral wall of CRT hearts supports this mechanism. This change is supported by recent human data reporting lower LV TNF-α (localization was not defined) after 6 months of CRT.

More striking than regional modifications by CRT was the decline in global apoptosis and enhanced cell-survival signaling. Reduced LV TUNEL positive staining also has been found in humans treated with long-term CRT, and the present findings reveal key pathways likely involved. The fall in Akt activity in DHF hearts is consistent with an earlier study using tachypacing-induced heart failure, with dysynchrony generated by right ventricular pacing. The presumption was that lower pAkt was due to cardiac failure itself; however, our data suggest that it may be more specific to dysynchrony because pAkt recovered with CRT even though hearts had persistent failure. The global distribution of altered Akt activation supports a more general impact of CRT, perhaps on mechanoenergetics.

Among the major targets of Akt that modulate cell survival, BAD phosphorylation best correlated. BAD is a member of the Bcl-2 family, which normally interacts with and suppresses antiapoptotic proteins Bcl-2/Bcl-X located at the mitochondrial membrane. This action depends on its phosphorylation state, and 14-3-3 modulation. BAD also is apoptosis. The present study supports an important role of BAD modulation by CRT, revealed by differential phosphorylation, mitochondrial levels, and 14-3-3 modulation. BAD also is phosphorylated at S112 by several kinases, including p90RSK, which could increase BAD phosphorylation. Although this site was also preferentially phosphorylated by CRT over DHF, p90RSK did not appear to be the culprit. One alternative is mitogen and stress kinase-1 linked to p38 activity, although our finding that p38 phosphorylation was lowered by CRT suggested that this is unlikely. Rather, we found that the major BAD phosphatase PP1α was reduced in CRT hearts, which could increase BAD phosphorylation.

Total BAD in cell lysates declined in both DHF and CRT hearts. This was not due to transcriptional regulation at the mRNA level, and intriguingly, mitochondrial BAD rose with
DHF (not CRT). Analogous disparities between total cellular and mitochondrial protein levels for Bcl-2 also were observed. The markedly reduced BAD phosphorylation with DHF would support relative Bcl-2 inhibition and a proapoptotic effect. Little is known about how BAD expression and Bcl-2 protein expression are regulated, although oxidative stress and hypoxia, coupled with opening of a mitochondrial ATP-sensitive potassium channel, may contribute. Whether such mechanisms apply to DHF or CRT remains to be determined. Finally, it is intriguing that phosphorylation of 2 other regulators of apoptosis that are inactivated by Akt (ie, antiapoptotic effect)—FOXO3α and GSK3β—did not correlate with Akt activity or CRT antiapoptotic effects. Signaling by these proteins, however, is complex, and their changes could reflect other influences (ie, worse failure with DHF) and phosphorylation by alternative kinases such as protein kinase A, protein kinase C, and MAPK.

Our study has several limitations. The model requires continued tachypacing to maintain heart failure while concomitantly applying resynchronization to examine its impact on the pathophysiology. Although different from what is found in the typical clinical setting, this model has advantages in that it helps isolate the influence of resynchronization per se from more general heart failure influences. The similarity between the TNF-α and apoptosis in the present study and a previous human CRT study supports its clinical relevance. The model is nonischemic, and it remains unknown whether identical results would be observed with ischemic cardiomyopathy. However, CRT is effective in both ischemic and nonischemic cardiomyopathy, and regional stress effects from dyssynchrony and its improvement by biventricular pacing are similar in both forms of failure. Another limitation is that we could not directly test the role of a given molecular change given the lack of specific pharmacological inhibitors usable in vivo and difficulties in genetically targeting these pathways in the intact canine heart.

Conclusions
We demonstrated both localized abnormalities in stress-response enzymes and global changes in cell-survival signaling that develop in the LV myocardium of the dyssynchronous failing heart and find that they can be favorably affected by biventricular stimulation to resynchronize contraction. These findings correlated with a very modest improvement in global function, suggesting that they may reflect more primary CRT effects from enhanced contractile synchrony that contribute to the long-term benefits of this treatment.

Sources of Funding
This study was supported by National Institutes of Health National Heart, Lung, and Blood Institute grants PO1-HL071780 (Dr Kass and Tomasselli) and T32-HL07227 (Dr Kass and Helm), the Peter Beller Laboratory, and the Abraham and Virginia Weiss Professorship (Dr Kass).

Disclosures
Dr Lardo has received a research grant from and served as a consultant or on the advisory board for Boston Scientific/Guidant. The other authors report no conflicts.

References


**CLINICAL PERSPECTIVE**

Cardiac resynchronization therapy (CRT) is arguably the most important therapeutic advance in heart failure treatment since the turn of the 21st century. It is used in patients with discordant contraction from conduction delays that in turn depresses global function and efficiency and induces regional loading disparities. This can exacerbate molecular stress signaling and stimulate cell death. CRT improves chamber mecanoenergetics, but whether or how it affects molecular stress/survival signaling is unknown. Because this is nearly impossible to study in humans, we developed a canine model using left bundle-branch radiofrequency ablation and rapid atrial pacing (3 weeks) to generate dysynchronous heart failure, followed by rapid biventricular pacing (3 weeks) to offset dysynchrony (CRT). Controls were atrially tachypaced for 6 weeks. CRT slightly improved function, but both groups had persistent substantial heart failure. Yet, although dysynchrony amplified lateral wall activation of p38 mitogen-activated protein kinase, calcium-calmodulin–dependent kinase II, and tumor necrosis factor-alpha, these disparities were absent with CRT. Cell death globally rose in dysynchronous hearts but was diminished by CRT. This was linked to the activation of the serine-threonine kinase Akt and distal phosphorylation of apoptosis-regulating protein BAD, enhanced BAD–14–3–3 interaction, and reduced phosphatase P1P1α. Other Akt-coupled modulators of apoptosis (forkhead—FOXO-3α and glycogen synthase kinase 3β) were less involved. These findings represent the first detailed analysis of stress and cell-survival signaling in failing dysynchronous hearts and highlight the importance of synchronous contraction and the novel benefits of CRT. These mechanisms likely contribute to enhanced cardiac performance and outcome from this therapy.
Reversal of Global Apoptosis and Regional Stress Kinase Activation by Cardiac Resynchronization


Circulation. 2008;117:1369-1377; originally published online March 3, 2008; doi: 10.1161/CIRCULATIONAHA.107.706291

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2008 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/117/11/1369

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2008/02/29/CIRCULATIONAHA.107.706291.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org/subscriptions/