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-3α 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.9 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

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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.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 anteropical 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,23 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), dysynchrony was also measured from magnetic resonance–tagged images (GE Signa, 1.5 T, GE Healthcare) by the circumferential uniformity ratio estimation.11

To assess short-term cardiac effects of biventricular pacing in our model of DHF, a separate group of 5 animals was instrumented long term for pressure–volume analysis as previously described.23 Pressure-volume relations were measured after 3 weeks of dysynchronous 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.

Figure 1. A, Representative regional strain tracings from 3-week DHF and at 6 weeks in both the DHF (continued dyssynchrony) and CRT (biventricular pacing for the last 3 weeks) models. Stain is determined by tissue Doppler and shows early anterior contraction and reciprocal lateral stretch, followed by the opposite motions in later systole. Right, This persists in DHF ventricles but is restored to more synchronous contraction in CRT hearts. B, Difference in ventricular function between weeks 3 and 6 for DHF vs CRT. CRT markedly reduced the dyssynchrony index (l), reflecting a decline in variance of strain timing. CRT also improved ejection fraction (EF) compared with DHF (p=0.02) and raised stroke volume (SV) more than DHF (p=0.058), and a trend toward less rise in end-systolic volume (ESV) was present. LBBB indicates left bundle-branch block; RAP, right atrial pressure; and EDV, end-diastolic volume.

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–conjugated secondary antibodies for 2 hours at 20°C. Protein was detected by chemiluminescence (ECL, Amersham BioSciences, Piscataway, NJ), and autoradiograms were analyzed with ImageJ software (National Institutes of Health, Bethesda, Md). Antibodies used were as follows: total Akt, P-Akt (T308), P-Akt (S473), p85, extracellular signal-regulated kinase (ERK1/2), Jun n-terminal kinase (JNK), Bel-2 antagonist of cell death (BAD) and phosphorylated BAD (pBAD; S136, S112), caspase-3, caspase-9, caspase-12, and PARP, ADP-ribose polymerase and caspase-3 (all at 1:1000, Cell Signaling Technology), and PP1-ADP-ribose polymerase (PARP), caspase-3 (all at 1:1000, Cell Signaling Technology), and P1-α (1:500, Santa Cruz Biotechnology, Santa Cruz, Calif), GAPDH (1:10000, ImageX, San Diego, Calif), and GAPDH (1:10000, ImageX, San Diego, Calif). GAPDH was probed in each membrane as a loading control. Displayed results are from 6 to 8 different dogs in each experimental group, with immunoblots performed in duplication and results averaged. Myocardial tumor necrosis factor-α (TNF-α) was assayed by quantitative ELISA (Quantikine, R&D Systems, Minneapolis, Minn) and spectrophotometry (M5, Molecular Devices, Carlsbad, Calif). Enzymatic activity assays were performed for p38 mitogen-activated protein (MAP), CaMKII, and Akt kinases (CycLex CY1177, CY1173, and CY-1168; MBL International) following the manufacturer’s instructions.

Analysis of Apoptosis

Ventricular myocardial apoptosis was assessed using several complementary methods. First, we assessed terminal deoxynucleotide transferase–mediated dUTP nick-end labeling (TUNEL) in formalin-fixed myocardial samples (CardioTACS In Situ Apoptosis Detection Kit,
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 (Fenia 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 resonance images.

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

<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 (A00)</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 (BiV-VOO)</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>
</tbody>
</table>

P < 0.004 0.8 0.4 0.02 0.06

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 VOO 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 CRT and †P<0.05 vs DHF group at 3-week time point. P<0.01 vs DHF and †P<0.01 vs CRT at 6-week time point. ‡P<0.001 vs DHF at 6-weeks.

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>Group</th>
<th>Dysynchronous (AOO)</th>
<th>Biventricular Pacing (VOO)</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.

Figure 2. A, Representative immunoblots for localized expression of p38 MAPK and CaMKII in lateral (Lat) and septal (Sep) regions of DHF and CRT hearts. Increased lateral wall expression declined with CRT. Summary data normalized to lateral wall expression are shown on the right. *P<0.05 vs lateral wall. B, Enzyme activity analysis for p38 MAPK and CaMKII confirms relative increases in the lateral wall in DHF hearts that decline with CRT. †P<0.001 vs control and CRT, P<0.05 vs septum; *P<0.001 vs control; †P<0.05 vs septum, P<0.05 vs CRT. C, Myocardial TNF-α assessed by ELISA (n=3, performed in duplicate; *P<0.05 vs control; †P<0.05 vs septum; P<0.05 vs CRT). Ep indicates epicardium; En, endocardium.

Figure 3. A, Representative immunoblots for localized expression of p38 MAPK and CaMKII in lateral (Lat) and septal (Sep) regions of DHF and CRT hearts. Increased lateral wall expression declined with CRT. Summary data normalized to lateral wall expression are shown on the right. *P<0.05 vs lateral wall. B, Enzyme activity analysis for p38 MAPK and CaMKII confirms relative increases in the lateral wall in DHF hearts that decline with CRT. †P<0.001 vs control and CRT, P<0.05 vs septum; *P<0.001 vs control; †P<0.05 vs septum, P<0.05 vs CRT. C, Myocardial TNF-α assessed by ELISA (n=3, performed in duplicate; *P<0.05 vs control; †P<0.05 vs septum; P<0.05 vs CRT). Ep indicates epicardium; En, endocardium.
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 but not CRT hearts (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.3 Figure 3A shows TUNEL staining in myocardium from control, DHF, and CRT hearts;
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.

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 but returned to control levels with CRT (Figure 5A). Total Akt was unaltered, but pAkt markedly declined in DHF vs CRT hearts (summary data on the right; n=6 to 8; \( P<0.001 \) vs DHF; \( P<0.01 \) vs CRT). Akt activity was further tested by an in vitro assay, which confirmed the pAkt expression findings.

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,25 but on phosphorylation, BAD dissociates and binds to 14-3-3 protein, suppressing apoptosis.26 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.27 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.

BAD also can be phosphorylated at S112 to confer antiapoptotic effects. Phosphorylation at this site was depressed in
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 MAPKs28 and protein kinase C.29 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.30,31 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.12 This was not observed in synchronous heart failure, normal hearts, or nonfailing dys-synchronous hearts,24 the last case highlighting the importance of combining heart failure pathophysiology with dis-coordinate 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 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 important given the impact of these proteins on muscle function, survival, and fibrosis. 

![Figure 6](https://example.com/figure6.png)

**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.

BAD also is phosphorylated at S112 by several kinases, including p90RSK. When phosphorylated by Akt (or p90RSK), BAD dissociates from the complex, binds to 14-3-3 protein, and suppresses 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 linked to the activity of ERK1/2 and protein kinase C. 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 activation 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.

![Figure 7](https://example.com/figure7.png)

**Figure 7.** A, Phosphorylation of BAD at S112 declined with DHF but was restored toward control by CRT (*P*<0.005 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).

...trigged 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 dyssynchrony 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 dyssynchrony 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 mecanoeenergetics.

...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 overexpression induces dilated cardiomyopathy. It can be...
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.

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Disclosures
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References
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During apoptosis, 14-3-3 protein by caspase-3 facilitates bad interaction with Bcl-x(L) during apoptosis. J Cell Biol. 1997;138:77–89.

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