Modulation of Mitochondrial Proteome and Improved Mitochondrial Function by Biventricular Pacing of Dyssynchronous Failing Hearts

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Running title: mitochondria alterations with CRT


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Abstract

Background - Cardiac resynchronization therapy (CRT) improves chamber mechanoenergetics, and morbidity and mortality, of patients manifesting heart failure with ventricular dyssynchrony, yet little is known about the molecular changes underlying CRT benefits. We hypothesized that mitochondria may play an important role, due to their involvement in energy production. Methods and Results - Mitochondria, isolated from the left ventricle in a canine model of dyssynchronous (DHF) or resynchronized (CRT) heart failure, were analyzed by a classical, gel-based, proteomic approach. Two-dimensional gel electrophoresis (2DE) revealed that 31 mitochondrial proteins where changed when controlling the false discovery rate at 30%. Key enzymes in anaplerotic pathways, such as pyruvate carboxylation and branched chain amino acid oxidation, were increased. These concerted changes, along with others, suggested that CRT may increase the pool of Krebs cycle intermediates, and fuel oxidative phosphorylation. Nearly 50% of observed changes pertained to subunits of the respiratory chain. ATP synthase beta subunit of complex V was less degraded and its phosphorylation modulated by CRT, associated with increased formation (2-fold, p=0.004) and specific activity (+20%, p=0.05) of the mature complex. The importance of these modifications was supported by coordinated changes in mitochondrial chaperones and proteases. CRT increased the mitochondrial respiratory control index with tightened coupling when isolated mitochondria were re-exposed to substrates for both complexes I (glutamate and malate) or II (succinate), an effect likely related to ATP synthase subunit modifications and complex quantity and activity. Conclusions - CRT potently impacts both the mitochondrial proteome, and performance associated with improved cardiac function.

Key words: cardiac resynchronization therapy, mitochondria, proteomics, ATP synthase
Introduction

Heart failure (HF) is a leading cause of morbidity and mortality in older adults, affecting 5 million individuals in the US alone\(^1\). A subset of HF patients also develops regional conduction delay, resulting in discoordinate contraction that worsens symptoms and ultimate prognosis.

Since the turn of the millennium, cardiac resynchronization therapy (CRT), also referred to as bi-ventricular (BiV) pacing, has become a clinical treatment for such patients, improving heart function, clinical symptoms and survival\(^2,3\). Some recent studies have revealed changes in gene expression, and molecular remodelling of stress response kinases and cell survival signaling\(^4,6\) associated with CRT. Among the earliest findings in clinical CRT studies was the demonstration that it improves chamber energetic efficiency. This is a rapid effect, resulting from the re-timing of contraction to occur synchronously in both sides of the myocardium, reducing wasted chamber work much like tuning a car engine. Given the importance of abnormal mechano-energetics in heart failure and centrality of ATP cycling\(^7\), we hypothesized that CRT may also strongly modify the underlying mitochondrial machinery responsible for energy supply.

Using a recently developed and reported canine model of dyssynchronous heart failure (DHF) and its resynchronization\(^5\), we determined protein changes in the mitochondrial sub-proteome by means of two-dimensional gel-based (pH 4-7 and 6-11) proteomics and additional detailed analysis of ATP synthase, the complex responsible for ATP production. Our findings are consistent with CRT acting as a metabolic therapy, increasing the abundance of enzymes that replenish the pool of Krebs cycle intermediates, reducing equivalents and subunits/mature complexes of the respiratory chain. We further demonstrate, in dog heart, that CRT modulation of a novel ATP synthase beta subunit (ATP\(\beta\)) phosphorylation (T311) may play a role in the regulation of complex V activity, protein turnover and assembly.
Material and Methods

Animal Model

Adult mongrel dogs (n=9) underwent either DHF or CRT protocol as previously described8, 9. See online supplement for more details.

Mitochondrial Fraction Preparation for Proteomics

The mitochondria-enriched fraction was prepared as we reported previously, with minor modifications10, 11. A detailed protocol description is available in the online supplement.

Two-dimensional Gel Electrophoresis (2DE)

2DE was performed on immobilized pH gradient (IPG) 18 cm strips (GE Healthcare), pH ranges 4-7 and 6-11, in the first dimension. See online supplement for details.

Protein Identification by Mass Spectrometry

Protein spots whose density was significantly changed with CRT were excised from fresh gels and digested as described previously12. Extracted peptides were submitted to ESI-Q-TOF tandem mass spectrometry (MS/MS). See the online data supplement for details.

Phosphopeptide Analysis by Immobilized Metal Affinity Chromatography (IMAC)

Individual ATPβ spots separated by 2-DE underwent in-gel trypsin digestion followed by extraction of the resulting peptides, as described in online supplement. Phosphopeptides were enriched with an immobilized metal ion affinity chromatography (IMAC) column essentially as described by Ficarro et al., with minor protocol modifications12, 13 described in the online
supplement. The reported phosphopeptide sequence was confirmed by manual inspection of the MS/MS spectra.

ATPβ De-Phosphorylation Assay

The phosphorylation status of ATPβ protein on 2DE gel was monitored using a modification of a previously described protocol\textsuperscript{14,15} (see online supplement for details).

Characterization of Isolated Complex V

Complex V was monitored by means of Blue Native-PAGE (BN-PAGE) as described in detail in the online supplement. The presence of ATPβ in F\textsubscript{1}/F\textsubscript{0} complex was assayed through western blotting using an anti-ATPβ antibody (MS503, Mitosciences, OR, US). BN-PAGE gels were used as well to measure the ATP synthase activity of complex V using the in-gel assay described by Bisetto et al.\textsuperscript{16} and in the online supplement.

Measurement of Oxygen Consumption in Isolated Mitochondria

Oxidative phosphorylation efficiency was measured on mitochondria freshly isolated from CRT and DHF animal hearts as described previously\textsuperscript{17}. Oxygen consumption was monitored at 37°C in the two groups, in the presence of either glutamate/malate or succinate as substrates, and after the addition of 300 μM ADP or 10 μM DNP to induce state 3 and maximal respiration, respectively. Additional details are provided in online supplementary methods.

2DE Data Analysis

Statistical significance of protein changes was assessed using two-sided two-sample t-tests, assuming equal within-group variability. p and q-values (for False Discovery Rate or FDR) are
provided in the text and Table 2. All statistical analysis was performed using the open source statistical environment R (http://cran.r-project.org). After statistical analysis a 1.5-fold cutoff was applied for biological significance18. See online supplement for more details.

Results

CRT Improves Cardiac Chamber Function

As previously reported, both groups of animals developed dilated cardiomyopathy, with reduced ejection fractions and elevated end-diastolic pressures. DHF hearts showed evidence of dyssynchrony (variance of peak systolic strain time around the heart 68±4.6 versus 31.3±5.1 in CRT (normal level is ~30; p<0.001). Animals assigned to CRT (bi-ventricular pacing) had improved ejection fraction and contractility measured at week six when compared with those maintaining dyssynchronous HF (DHF) (Table 1). Paired data between the third and sixth week time points have confirmed improved function in CRT and reduced function in DHF5. Table 1 summarize functional data recorded from DHF and CRT animals prior to sacrifice.

Protein Changes with CRT

Roughly 1200 protein spots were visible after silver staining on high resolution 2DE gels in the 4-7 and 6-11 pH ranges (Figure 1). Thirty one quantitative protein changes were observed in isolated mitochondria from CRT vs. DHF hearts when controlling the FDR at 30% (and 21 when controlling at 20% FDR, see Table 2 for q-values). Representative spot images and quantification of the various protein spots that changed between DHF and CRT are shown, clustered in functional groups, in Figure 2. Protein spot identifications, together with 2DE and MS/MS data, fold changes, and statistics are listed in alphabetical order in Table 2 and numbered accordingly, together with the protein accession number as provided by NCBI non
redundant protein database (“other-mammals” sub-database, see online section for Table 2 description). Albeit 2DE is historically known for its bias against membrane proteins\textsuperscript{19}, our optimized separation protocol\textsuperscript{10} allowed us to obtain extensive representation of the mitochondrial proteome, including a large number of basic and membrane proteins (see Channels and mitochondrial membrane proteins section).

\textit{Respiratory Chain}

Approximately half of the observed mitochondrial protein changes (15 out of 31) pertained to the respiratory chain, consistent with CRT modulating ATP production (Figure 2A). Changes were observed in all protein complexes of the respiratory chain, with the sole exception of complex IV. These included complex I 1\textalpha subcomplex 6, 14 kDa, Fe-S Protein 1, 75 kDa and B16.6 which are increased (2 to 5-fold). The enzymatic complex linking the Krebs cycle to respiratory chain, complex II succinate dehydrogenase (SDH), increased in the CRT mitochondria (2-fold) and was phosphorylated (see Supplementary Figure 1A and B), and one complex III subunit (binding protein) increased in CRT compared to DHF (3-fold). Interestingly, two different protein spots with different pIs were identified as cytochrome c; all of these exhibited a decrease in spot density in the bi-ventricular pacing group (-2-fold,). Complex V is also changed, and given the importance of its modifications we characterized it in greater detail (see below).

\textit{Metabolic Pathways}

Metabolic pathways that supply intermediates for the Krebs cycle (presented in Figure 2B) were all increased by CRT. Changes were observed in pyruvate carboxylase (3-fold) and pyruvate dehydrogenase, E1 (>10-fold) and E2 (2-fold) subunits. Additional key enzymes which fuel the
Krebs cycle were also increased (Figure 2C): aldehyde dehydrogenase (2-fold), alpha-keto acid dehydrogenase E2 (2-fold), and ferredoxin reductase (2-fold). Fatty acid binding protein, which participates in fatty acid transport into the mitochondrial matrix for final oxidation, was also increased by CRT (4-fold).

Protein Synthesis/Import

Proteins that participate in mitochondrial protein synthesis/import to mitochondria (Figure 2D) were mainly up-regulated with one of the more prominent changes involving Prohibitin 2 (5-fold). It is noteworthy that the Prohibitin 2 protein spot (pI=10.04) is perfectly resolved, an uncommon observation for such an extremely basic protein. Leucine-rich PPR motif-containing protein was also increased (2-fold). The 28S mitochondrial ribosomal subunit (MRP-S22) declined in the CRT group (-1.5-fold).

Channels and Mitochondrial Membrane Proteins

Two isoforms of the voltage-dependent-anion-channel (VDAC2 and 3) showed a different abundance with CRT (-2-fold and 3-fold respectively) indicating that the solubilization and separation methods utilized are effective in recovering and resolving very basic and hydrophobic proteins (Figure 2E; also refer to Table 2 for predicted pI values). As well, a more acidic form of another mitochondrial membrane protein of unknown function, XP_533991, is recovered in the basic gels, and decreased with CRT (-2-fold) (Figure 2F).

ROS Scavenging

The reactive oxygen species (ROS) scavenging apparatus (Figure 2G) in the mitochondria was augmented by CRT, as reflected by increased levels of both thioredoxin-dependent peroxide
reductase (>10-fold change) and of a modified form (based on experimental pI deviation from predicted one) of the programmed cell death 8 protein (AIF) (2-fold) (Figure 2G). This and other protein changes (Figures 2H and 2I) are discussed in the online supplement (Supplemental Results and Discussion sections).

*Modifications of ATP synthase*

ATP synthase beta subunit (ATPβ), part of respiratory chain’s complex V, was assigned to 6 protein spots. Since all of them displayed an experimental MW lower than the theoretical one they are likely fragments of ATPβ. All were decreased (ranging from -3-to-2 fold) (Figure 3A and Supplementary Figure 2). Because samples were kept on ice during mitochondrial enrichment, and processed under identical conditions, it is unlikely that artifactual protein degradation occurred. Furthermore, mitochondrial samples left on ice for eight hours (sampled every two hours) were subjected to 1DE separation and western blot analysis, and no evidence of artifactual ATPβ protein degradation was discerned (data not shown).

In order to investigate the contribution of phosphorylation to ATPβ protein spot changes we used a 2DE-DIGE-based method, in which endogenous samples (DHF and or CRT) were pooled and dephosphorylated with alkaline phosphatase and used as an internal standard (de-phosphorylated proteome). Figure 3B shows the 2DE separation of ATPβ obtained before and after de-phosphorylation. The cluster of coloured spots correspond to differently phosphorylated forms of ATPβ: due to CyDyes colour assignment, the protein spots with a dominant red colour (Cy3) are more abundant in DHF, whereas green colour (Cy5) characterizes spots that are more abundant in the CRT group. The blue channel (Cy2) detects the “de-phosphorylated” proteome, so stronger blue colour indicates less- or un-phosphorylated protein, confirming the presence of phosphorylation in adjacent acidic spots. Protein phosphorylation can affect electrophoretic
mobility in two ways. The addition of a phosphate group can shift the pI value of the protein towards the acidic end; but can also produce an apparent increase in the molecular weight by probably affecting protein-SDS interaction (due to negative charge). Therefore, un-phosphorylated (or less–phosphorylated) ATPβ protein spots appear at the bottom-right in panel B compatible with a more basic pI and lower molecular weight after phosphate groups removal by alkaline phosphatase (AP). It is interesting to note that just a small fraction of the subunit is not phosphorylated (intact form, Figure 3, 2-fold increased, p=0.048, q=0.31 ). This suggests that very little of the subunit exist in an un-modified form, in vivo, and that PTMs probably play a major role in regulating its biological function. We also found that another complex V subunit, ATPδ, was phosphorylated, but similarly in both DHF and CRT (see Supplementary Figure 1C and D).

To characterize ATPβ protein phosphorylation in more detail, IMAC was used upstream of MS. MS/MS spectra of ATPβ peptide FTQAGSEVSALLGR (m/z=709.4) is shown in Figure 3D. De novo sequencing confirmed the presence of a phosphate group based on b10 and b12 ions. The only phosphorylatable amino acid residue within this sequence is the Threonine residue (T311). Interestingly the homologue position in rabbit sequence was found to be phosphorylated in rabbit heart upon pharmacological preconditioning with adenosine12.

The complex assembly and degradation of ATP synthase is highly regulated. The effect of CRT on ATPβ protein inclusion into mature multimeric complexes (F₁/F₀) was monitored through BN-PAGE20. Mild detergent conditions, which still allowed separation of native complexes, were used in the electrophoretic separation. Figure 3C shows a western blot analysis of a non-denaturing BN-PAGE gel where a specific antibody for ATPβ was used. The F₁/F₀ of complex
V was detected (a western blot, using anti-ATP synthase α subunit antibody, produced the same immuno-pattern; data not shown). The F₁ is membrane-associated whereas the F₀ is the oligomycin-sensitive transmembrane portion; the F₁ classically consists of 3 α, 3 β and 2 δ subunits. The intact F₁/F₀ was increased in CRT group (2-fold, p=0.004 vs. CRT) indicating an increase of the mature, assembled complex V with CRT. Interestingly, the same form of the complex was not significantly changed in DHF compared to sham operated animals (Figure 3C).

**Mitochondria Coupling Improves with CRT**

The efficiency of oxidative phosphorylation was determined in mitochondria isolated from shams and dogs subjected to DHF and CRT (Figure 4). In DHF dogs, mitochondrial oxygen consumption was significantly increased in the basal state (state 4) as compared to shams or CRT, in the presence of both complex I substrate glutamate/malate (Figure 4A) and complex II substrate succinate (Figure 4B). However, respiration in state 3 and uncoupled state, induced by addition of ADP and protonophore DNP respectively, did not differ between the groups. CRT prevented mitochondrial uncoupling, thus enhancing the respiratory control ratio over DHF (Figure 4C). ADP/O ratio (Figure 4D), an index of the oxidative phosphorylation efficiency, was significantly greater in CRT and shams when compared to DHF mitochondria.

Finally, the intrinsic activity of complex V, isolated in native conditions according to Bisetto et al. 2016, was increased by 20% with CRT compared with DHF (p=0.05) (Figure 4E) whereas no significant difference was observed between DHF and sham animals. The activity was measured as the density of lead phosphate bands generated after incubation in the presence of ATP, normalized to the quantity of complex V (F₁/F₀), and therefore it can be considered as the “specific activity” of the complex (see also online supplement).
4. Discussion

The present study explored the mitochondrial sub-proteome of dyssynchronous and re-synchronized (BiV paced) failing hearts, employing an improved separation methodology for basic proteins\textsuperscript{10}, a modified version of de-phosphorylation 2DE (combining DIGE and alkaline phosphatase treatment)\textsuperscript{14}, and a phosphopeptide enrichment MS-based method for phospho-residues identification\textsuperscript{22} all of which were coupled with functional targeting of complex V. We identified 31 mitochondrial proteins as well as two mitochondria associated proteins that were changed in the CRT group compared to DHF controlling at 30\% FDR. These are summarized in a schematic shown in Figure 5. The changes encompass many facets of mitochondrial function, including: metabolic pathways, ATP production, protein turnover, oxidative stress, and apoptosis. The results were also analysed using the Ingenuity Pathways Analysis tool which confirmed the significance regulation of “mitochondrial dysfunction” and “oxidative phosphorylation” canonical pathways (Supplementary Figure 3).

The most important observation of our study is the modulation of metabolic pathways and ATP production machinery in CRT treated hearts. Specifically, CRT appears to broadly influence metabolic pathways that regulate pyruvate metabolism. Pyruvate carboxylase (PC) was notably increased with CRT. PC is a key anaplerotic enzyme, important for replenishing the Krebs cycle. Intracoronary administration of pyruvate to heart failure patients acutely improves hemodynamics and cardiac performance, and this inotropic effect is thought to be due mostly to its effects on the Krebs cycle through pyruvate dehydrogenase (PDH) and other anaplerotic pathways\textsuperscript{23}. In the heart, the concentration of Krebs cycle intermediates (KCi) is critical and the depletion of carbon flux into the cycle results in contractile dysfunction\textsuperscript{24}. It is likely that increased levels of PC provide a supplementary source of KCi. Carbon units traditionally enter the Krebs cycle through PDH. The observed increase in the E1 and E2 subunits of PDH likely
underlies a parallel effect on KCi formation. Both pathways improve the utilization of pyruvate, and the consequent formation of reducing equivalents (NADH, FADH2) which are later oxidized by the electron transport chain.

Another important metabolic pathway in the heart relates to the branched-chain amino acids (BCAA: Val, Leu, Ile) oxidation since carbon derived from BCAA can also replenish the KCi pool via formation of acetyl-CoA. Benefits from BCAA utilization, in isolated normal and septic hearts have long been recognized\textsuperscript{25, 26}. Intriguingly, we found that the E2 subunit of branched chain alpha-keto acid dehydrogenase was increased in CRT compared to DHF. CRT also enhanced the abundance of fatty acid-binding protein (FABP), a well established regulator of beta-oxidation which fluctuates in mitochondria along with peaks in beta-oxidative activity\textsuperscript{27}. Heart metabolism in hypertrophic and failing hearts may shift from fatty-acids to glucose utilization\textsuperscript{28}; and in this light an increase in FABP might reflect the reversal to a more normal and efficient metabolic phenotype. This view is also supported by recent metabolomic studies.

Turer and colleagues recently showed that Ischemia/Reperfusion injury decreases the oxidative fuel metabolism (fatty acids and ketone bodies) in favour of an increased glucose metabolism\textsuperscript{29}. As well, Mayr and co-workers recently analyzed the metabolomic and proteomic changes associated with human atrial fibrillation (AF) and found alterations of glycolytic enzymes, in the absence of a significant increase of glucose metabolites. Also, the transcriptomic, proteome and metabolomic data pointed towards an inhibition of PDC complex during AF, in agreement with our observations\textsuperscript{30}. Finally, the changes in metabolic protein are supported by a recent transcriptomics study from our group\textsuperscript{6}. 
Succinate dehydrogenase (SDH) was increased in CRT versus DHF, suggesting that augmented availability of KCi may fuel the respiratory chain via complex II. While optimizing the ATPβ de-phosphorylation assay, we also found that SDH is heavily phosphorylated in this canine pacing model (Supplementary Figures 1A and B). Moreover, the pI and MW co-ordinates of the SDH protein spot increased with CRT indicate that this is the un-phosphorylated, precursor form of the flavoprotein subunit of complex II and suggest that the observed increase was likely due to newly synthesized protein. The additional observation that isocitrate dehydrogenase activity is increased (Supplementary Figure 4) provides functional evidence that Krebs cycle activity was stimulated with CRT.

More than half of the observed protein changes resided in the respiratory chain. Generally, subunits of complexes I, II, III and V were increased upon CRT, suggesting a compensatory shift towards an increased ability to produce ATP through oxidative phosphorylation. As such it is surprising that two forms of cytochrome C (cytC) were decreased; and according to the difference between the observed and predicted pI/MW of the protein, these two forms likely represented modified forms of cytC. In the present study we were unable to observe a shift in the pI of cytC after treatment with alkaline phosphatase coupled with western blot analysis. This either suggests that: a) the observed pI shift is due to an unknown post-translational modification (PTM); b) cytC is phosphorylated but the antibody used did not recognize this form of the protein; or c) alkaline phosphatase could not de-phosphorylate cytC efficiently. Indeed, alkaline phosphatase shows a low specificity by nature, and the assay conditions were optimized for ATPβ de-phosphorylation. However, in general, the a-specificity of AP represents an advantage rather than a limit, at least in broad-based screening de-phosphorylation assays, such as those required in proteomic analyses15.
The ATPβ subunit of complex V was less proteolyzed and its phosphorylation status altered with CRT, the quantity of the mature F₁/F₀ complex increased two-fold and its specific activity increased by 20%. This suggests a novel mechanism for complex V activity regulation that acts both on complex V assembly and specific activity. Indeed, CRT not only increases complex V assembly but this is 20% more active. Interestingly, along with reduced degradation of ATPβ in CRT, Prohibitin 2 (Phb2) increased. Phb2 is a chaperone known to regulate mitochondrial protein stability and turnover and the deletion of prohibitin genes in yeast causes an increased degradation of mitochondrial membrane proteins by the membrane embedded m-AAA protease system. Augustin et al. reported the existence of a constant efflux of peptides derived from mitochondrial protein degradation into the cytosol and regulated by AAA-proteases and chaperones such as the prohibitins. Using mass spectrometry, the authors estimated that more than half of these peptide species were derived from the electron transport chain proteins. The decrease of mAAA-proteases such as ClpP with CRT, supports this view and suggests that ClpP is a potential candidate for the proteolysis of complex V observed in the present study. When we performed a 2DE western blot analysis for ClpP we found two forms, with the same pI but different molecular weight (≈10³ Da, see Supplementary Figure 5). The higher molecular weight form is reduced, in agreement with differential display analysis whereas, the lower molecular weight form is increased with CRT.

This study also identified a novel phosphorylated amino acid residue of ATPβ isolated from the DHF heart (T311). Threonine 311 is homologous to a site previously identified by our group in the heart of rabbits subjected to adenosine–induced preconditioning. We believe that this is an
important discovery since the site-specific characterization of PTM could help identify candidate kinases and other enzymes based on their consensus sequence.

The functional role of this phosphorylation is unknown, and mutational studies to assess this are in progress; but it is tempting to speculate that phosphorylation may be involved in ATP synthase protein folding, activity and turn-over regulation. F$_1$/F$_O$ complex assembly seems to be affected by phosphorylation mechanisms, at least in yeast where it has been extensively studied. These studies suggest that phosphorylation-induced conformational changes expose proteases consensus sequences that would be normally buried in the un-phosphorylated protein$^{31}$. This view is in agreement with the increased formation of assembled F$_1$/F$_O$ subunits with CRT, as shown by BN-PAGE in the present study. The mechanism of assembly of complex V subunits into F$_1$ and F$_O$ sectors is still the object of intense research and a role for complex V modifications and assembly in mitochondrial uncoupling has also been reported$^{33}$. Yeast mutants lacking ATP$_{\gamma}$ ($\gamma$-less yeast) grow very slowly on fermentable carbon sources and this has been linked to a proton leak through complex V. In native conditions, the inhibitor protein (Inh1p) prevents ATP synthase from working in reverse, as an ATPase. In $\gamma$-less yeast, Inh1p cannot as efficiently bind complex V and the complex dissipates protons and loosens mitochondrial coupling$^{33}$. We report an increase in the respiratory control index, indicating a tighter mitochondrial coupling with CRT. This observation, together with the changes in ATP$\beta$ modifications and the improved activity and assembly of complex V with CRT, suggests that complex V is not only adapting but probably amplifying the beneficial effects of CRT on the up-stream pyruvate metabolism. Our group is presently investigating the causal relation between ATP$\beta$ modifications and complex V assembly and activity, which lies beyond the scope of the present study. Moreover, the observation that the quantity and activity of mature complex V is not changed in DHF hearts when compared to shams suggests that the beneficial effects of CRT
rely on a compensatory response that involves energetics rather than a reversal to the sham phenotype.

5. Conclusions

We have shown that resynchronization by BiV pacing of a dyssynchronous failing heart alters the expression and post-translational status of many mitochondrial enzymes that fuel pyruvate metabolism, and therefore the respiratory chain, the site of ATP production. Notably, we demonstrate that subunits of Complex V (ATP synthase) are less degraded (ATPβ and α) and differentially phosphorylated (ATPβ) with CRT. The specific activity and the quantity of mature complex V, measured in native conditions, are increased as well. These and other observed changes suggest that CRT acts at several co-ordinated levels of the mitochondrial proteome: the pyruvate metabolism, the Krebs cycle, and the respiratory chain subunits, with targeted modifications and increased activity and assembly of complex V, resulting in the overall improvement of mitochondrial function. The data support the notion that in addition to enhancing mechanical efficiency at a chamber level, CRT potently alters cellular energy metabolism in the mitochondria. This may play an important role in the ability of CRT to enhance the systolic work performance of the failing heart acutely and chronically while also improving long-term survival - something not yet achieved by pharmacologic therapies.

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Conflicts of Interest Disclosures

Dr. J.E. Van Eyk and Dr. D.A. Kass are consultants for Boston Scientific Research (modest and significant, respectively). Dr. D.A. Kass and Dr. G.F. Tomaselli are recipients of funding from Boston Scientific Research (significant).

References


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<td>gi</td>
<td>74003189</td>
</tr>
<tr>
<td>Programmed cell death 8 isoform 2 isoform 6 (AIF)</td>
<td>20</td>
<td>gi</td>
<td>74008419</td>
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<tr>
<td>Prohibitin 2</td>
<td>21</td>
<td>gi</td>
<td>76016402</td>
</tr>
<tr>
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<td>73982897</td>
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<tr>
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<td>23</td>
<td>gi</td>
<td>76600371</td>
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<tr>
<td>Pyruvate dehydrogenase E2 (PDH)</td>
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<td>73954761</td>
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<tr>
<td>SAM50-like protein CGI-51</td>
<td>25</td>
<td>gi</td>
<td>73969335</td>
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<tr>
<td>Succinate dehydrogenase</td>
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<td>gi</td>
<td>74003064</td>
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<tr>
<td>Thioredoxin-dependent peroxide-reductase</td>
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<td>gi</td>
<td>73998671</td>
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<tr>
<td>Ubiquinol-cytochrome C reductase binding protein</td>
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<td>gi</td>
<td>73999619</td>
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<tr>
<td>XP_533991</td>
<td>31</td>
<td>gi</td>
<td>73987821</td>
</tr>
</tbody>
</table>

**Mitochondria associated proteins:**

- **Desmin**
  - A | gi|59958381 | 28 | 2063 | 5.3/53 | 5.21/53 | 2.1 | 0.003 | 0.10 |
  - B | gi|65987 | 7  | 504 | 7.6/38 | 6.90/36 | 2.0 | 0.023 | 0.23 |

*NOTE:* two level of stringency were applied for both statistical (p<0.05, q<0.3 or 30% FDR) and biological (1.5-fold change) significance. Proteins with q-values above 0.2, which normally wouldn’t merit claims of signals were retained for their “biological coherence” (eg. subunits of the same complex, see online supplement).
Legends to Figures

**Figure 1.** Mitochondrial proteome. Representative silver stained 2DE gels on two pI ranges: 4-7 and 6-11. pI (based on linear distribution) and MW (based on MW markers) values are shown on the top and the sides respectively. Proteins are numbered according to Table 2.

**Figure 2.** Protein changes with CRT. Magnified spot images from CRT and DHF representative gels, are presented along with plots of their normalized, back-ground subtracted, volume. Protein spots are numbered according to Table 2. Protein names with grey backgrounds are those that increased with CRT.

**Figure 3.** Characterization of ATPβ PTMs. Distribution of changing ATPβ on the 2DE gels is shown in panel A. The un-modified form (marked with a thicker arrow) and fragments are indicated by arrows, and accompanied by fold changes values. Panel B shows a representative de-phosphorylation gel for ATPβ. DHF in red (Cy5), CRT in green (Cy3), and the AP-treated pool (AP) in blue (Cy2).

Panel C shows representative BN-PAGE/WB for ATPβ. Biological replicates are shown (3 sham operated controls, 5 DHF and 4 CRT). Panel D presents a typical MS/MS profile for the phosphopeptide FT*QAGSEVSALLGR. The MS/MS spectrum is provided, together with a schematic in which the mass values for al b-an y-ions series are reported.

**Figure 4.** Mitochondrial bioenergetic parameters. Mitochondrial oxygen consumption was measured in the presence of glutamate/malate (panel A) and succinate (panel B) as substrates. Respiration was monitored in: basal state, or state 4 (S4); state 3, induced by the addition of 300 μM ADP (S3); and uncoupled state, induced by the addition of protonophore DNP (10 μM,
UNC). Respiratory control ratios are shown in panel C and ADP/O ratios are plotted in panel D. (*p<0.05, **p<0.001 vs. CRT, ‡p<0.05 and ‡‡p<0.005 vs. shams). Panel E shows a representative image of the in-gel ATPase assay (n=3). Specific activity is reported as the density of white PbHPO₄ bands (product) normalized for the amount of complex V as indicated by coomassie blue G250 stain (*p=0.02 vs. shams and p=0.05 vs. DHF).

**Figure 5.** Schema of mitochondrial protein changes with CRT. For values, refer to Table 2. (pI, observed pI differs more than 1 pH unit from predicted one; MW, observed MW differs more than 10 kDa from predicted one).
Figure 2

A. Respiratory chain

Complex I

NADH dehydrogenase (ubiquinone)

1α subcomplex, 6,14kDa

Fe-S protein 1, 75kDa

NADH-ubiquinone oxidoreductase

B16.6 subunit;

Complex II

Succinate dehydrogenase

Cytochrome C

Complex III

Ubiquinol-cyt C reductase binding

B. Pyruvate Metabolism

Pyruvate Carboxylase

Pyruvate dehydrogenase E1

Pyruvate dehydrogenase E2

DHF CRT

Complex V, F₁

ATPα (fragment)

ATPβ (fragments)
C. Other metabolic pathways

Alcohol metabolism
- Aldehyde dehydrogenase

BCAA metabolism
- Alpha-keto acid dehydrogenase E2

Fatty acid metabolism
- Fatty acid-binding protein

Steroid metabolism
- Ferredoxin reductase

D. Mitochondrial protein synthesis/import
- Leu-rich PPR motif-rich prot.
- Mito. 28S ribosomal prot. S22
- Prohibitin 2

E. Channels
- VDAC2
- VDAC3

F. Membrane proteins
- XP_533991

G. ROS scavenging
- Thioredoxin-dep.-peroxide-reductase
- Programmed cell death 8 (AIF)

H. Mixed IDs
- NADH dehydrogenase Fe-S, 30kDa/ClpP
- SAM50-like ATP

I. Mitochondrial associated proteins
- Desmin
- GAPDH
Figure 3

A. Intact, unphosphorylated (+1.8, p=0.048, q=0.31)

Fragments (-2.9 to -1.8, 0.001 < p < 0.03, 0.04 < q < 0.27)

B. ATP

DHF

AP

DHF~Cy5 CRT~Cy3 AP~Cy2

C. ATPβ (BN-PAGE)

D. F1/F0

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Figure 4

A. GLUTAMATE/MALATE

B. SUCCINATE

C. Glutamate/Malate

D. Succinate

C.

<table>
<thead>
<tr>
<th></th>
<th>Glutamate/Malate</th>
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<tbody>
<tr>
<td>sham</td>
<td>4.0±0.3</td>
<td>2.9±0.45</td>
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<tr>
<td>CRT</td>
<td>5.2±0.5</td>
<td>3.9±0.5</td>
</tr>
<tr>
<td>DHF</td>
<td>1.8±0.03 **‡‡</td>
<td>1.6±0.4 *</td>
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</table>

E. In-gel ATPase assay

720 kDa

F1/F0

norm vol. %

<table>
<thead>
<tr>
<th></th>
<th>Glutamate/Malate</th>
<th>Succinate</th>
</tr>
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<tbody>
<tr>
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<td>DHF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRT</td>
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</table>

* ‡ Significance levels for comparisons between groups.
Modulation of Mitochondrial Proteome and Improved Mitochondrial Function by Bi-ventricular Pacing of Dyssynchronous Failing Hearts

Giulio Agnetti, Nina Kaludercic, Lesley A. Kane, Steven T. Elliott, Yurong Guo, Khalid Chakir, Daya Samantapudi, Nazareno Paolocci, Gordon F. Tomaselli, David A. Kass and Jennifer Van Eyk

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SUPPLEMENTAL MATERIAL

Supplemental Methods

Reagents

18 cm IPGstrips (pH range 4-7 and 6-11), agarose NA, and CyDyes were purchased from GE healthcare. Bis-acrylamide from BioRad Laboratories. Alkaline Phosphatase (Calf Intestinal Phosphatase, CIP) from New England Biolabs. Protease inhibitors Complete from Roche. Mark 12 (unstained) and Seeblue (prestained) molecular weight markers, precast NuPAGE (4-12% BisTricine) and Blu Native BN-PAGE gels and PVDF membrane (immobilon, Millipore) from Invitrogen. The anti-ATP synthase beta subunit antibody was purchased from MitoSciences (MS503, mouse, monoclonal). All other chemicals were purchased from Fischer Bioreagents, and were of the highest analytical grade.

Workflow

Supplementary Figure 6 describes the experimental strategies used in the present study. Altered proteins detected by spot density were identified using tandem mass spectrometry (MS/MS or tandem MS). Changes in the phosphorylation status of ATPβ and other proteins were monitored using a modified version of the protocol defined by Raggiaschi et al.1,2. The dephosphorylated pool used here in place of the internal standard should be common to both samples conditions, at least as far as phosphorylation status is concerned (see below). Phospho-peptide enrichment with IMAC was used in combination with MS/MS to improve identification of the exact phosphorylated amino acid residues. Finally, functional assays were used to characterize complex V activity and assembly along with the determination of respiratory control index in isolated mitochondria.
Animal Model

Five animals were paced from the right atrium for six weeks at ~200 bpm (DHF); whereas the remaining four dogs were subjected to three weeks of atrial pacing (dyssynchrony) followed by three weeks of bi-ventricular tachypacing at the same rate (CRT) as described in3. Left bundle branch block (LBBB) was confirmed by intra-cardiac electrograms, with surface QRS widening from 50±7 to 104±7 ms (p<0.001). Bi-ventricular pacing was achieved by simultaneous lateral epicardial and right ventricular antero-apical free wall stimulation. At terminal study the hearts were extracted under cold cardioplegia, dissected into endocardial and mid/epicardial segments from the septum (i.e. LV and RV septum) and LV lateral wall, and frozen in liquid nitrogen. Tissue samples obtained from the upper third of the LV lateral wall were used in the present study.

Mitochondria Fractions Preparation

The protocol was designed to optimize reproducibility, minimize contamination from other subproteomes (e.g. myofilaments), and maximize protein recovery. Samples were kept on ice throughout the entire process to minimize artifactual protein modifications.

Mitochondrial protein enrichment and myofilament contamination were monitored, along with the separation (Supplementary Figure 7). Briefly, frozen heart tissues (typically 500 mg) were crushed into a mortar filled with liquid nitrogen by means of a ceramic pestle. All of the following steps were performed in ice to prevent artefact protein degradation. Frozen tissue crumbs were transferred in a glass homogenizer filled with ice-cold H-buffer (220 mM mannitol, 70 mM Sucrose, 20 mM Hepes, pH 7.4) and briefly homogenized. Tissue homogenates were filtered through a 100 µm filter to remove connective tissues and collect
intact cardiac myocytes. The filtrate was centrifuged (1100 rcf, 5 min, 4 °C) and rinsed with ice-cold H-buffer four times. Supernatants were collected, and pellets discarded (cell debris, nuclei, and intact cells). Mitochondria were next “washed” from membranes and broken mitochondria by centrifuging three times at increasing centrifuge fields (7000, 18000 and 20000 rcf, 15 min, 4°C) and re-suspending the pellet in half of the volume after each centrifugation. Two further centrifugation steps (3000 rcf, 3 min, 4°C) were required to remove remnant myofilaments; supernatants were collected and finally centrifuged at high speed (20000, 20 min, 4°C) to pellet the purified mitochondria. Pellets were re-suspended in 100 μL H-buffer, gently, by pipetting up and down few times. 1 μL of the suspension was transferred into a 96 multiwell plate (flat bottom, costar) and diluted in 20 μL of 1% SDS, 5 % ASB 14 and protease inhibitor complete, in triplicate. A BSA (sigma, 2 mg/ml in the same buffer) standard curve was used to assay for protein quantity after 30 min incubation at 37°C (BCA, Pierce). Protein concentration was calculated, and mitochondrial suspensions were divided accordingly into 100 μg aliquots, and then flash frozen in liquid nitrogen for storage at -80°C. Mitochondrial protein enrichment and myofilament protein contamination was tested along with the purification process as displayed in Supplementary Figure 7.

Two-dimensional Gel Electrophoresis

Optimal resolution for basic mitochondrial proteins was achieved using the improved protocol developed by our laboratory. The second dimension (SDS-PAGE) was run using 10% bis-tris gels with 2(n-morpholino) ethansulfonic acid (MES) running buffer. Gel slabs were subsequently silver stained according to Shevchenko. Sample pellets were
thawed and centrifuged (20000 rcf, 20 min, 4°C) and supernatant (H-buffer) discarded. 5% amidosulfobetaine-14 (ASB-14, Calbiochem) was added to the sample to solubilize membrane proteins prior to dilution in isoelectric focusing (IEF) re-hydration buffer (8 mol/L urea, 2.5 mol/L thiourea, 4% w/v 3-[3-cholamidopropyl]-1-propane-sulfonate [CHAPS], 0.5% ampholytes, 50 mmol/L DTT, 1% HED, and 0.01% w/v bromophenol blue). IEF was carried out using a Protean® IEF cell (Bio-Rad). Immobilized pH gradient (IPG) Strips (18 cm pH 4-7 linear gradients) were actively rehydrated with the sample (150 µg of protein in 350 µL IEF buffer) at 50 V for 12 hrs, followed by a rapid voltage ramping consisting of 1 hr each at 300, 600, and 1000 V, followed by 10000 V for 45 kWh at 20°C. For pH 6-11 IPG strips, IEF strips were passively rehydrated before focusing (10 hrs). Protein samples were added to rehydration buffer to give a final protein concentration of 1 mg/mL, and applied by paper bridge-loading as described in4. Proteins were separated in the second dimension by 10% Bis-Tris SDS-PAGE, using a MES running buffer (45 mmol/L [2-(N-morpholino) ethane sulfonic acid] or MES, 50 mmol/L Tris base, 0.1% SDS, 0.8 mmol/L EDTA, pH 7.3) as described previously5. IPG strips were reduced and alkylated for 20 min each, respectively using 1% (w/v) DTT and 4% (w/v) iodoacetamide in equilibration buffer (50 mmol/L Tris-HCl, pH 8.8, 6 mol/L urea, 30% v/v glycerol, 9% w/v SDS). IEF strips were rinsed briefly with MES running buffer, the excess of liquid was gently removed with a paper tissue, and the strips were loaded onto the 10% Bis-Tris SDS-PAGE gels. Strips were sealed using agarose sealing solution (50 mmol/L MES, 0.5% Agarose NA, 0.1% w/v SDS, bromophenol blue). Gels were run overnight on a Protean® II XL system (Bio-Rad) at 90 V. Gels were silver stained according to the protocol of
Shevchenko et al.\textsuperscript{6}. Differential display analysis was performed using Progenesis (Nonlinear Dynamics, NC, US).

\textit{Immunoblotting}

Proteins were transferred to PVDF in transfer buffer at 100 V for 1 hour in ice. Membrane were stained with Direct Blue 71 (Sigma), and images recorded for subsequent luminescent signal normalization. Membranes were then blocked overnight using Western Blocking Reagent (Roche); and incubated with 0.2 $\mu$g/mL anti-ATP synthase beta subunit antibody mouse IgG monoclonal (MS503, MitoSciences) in Tris-buffered saline completed with Tween 20 (TBS-T: 100 mmol/L Tris-Cl, 0.9\% (w/v) NaCl, 0.1\% (v/v) Tween 20) under gentle agitation for 1 hr, and then incubated with 0.03 $\mu$g/mL alkaline phosphatase-conjugated AffiniPure Goat Anti-Mouse (Jackson ImmunoResearch) in TBS-T under gentle agitation for 1 hr. For ATP synthase delta 2D-WB a rabbit-polyclonal antibody was used (anti-ATP5D, Atlas/Sigma Prestige, 0.05 $\mu$g/mL in TBS-T). In this case a alkaline phosphatase-conjugated AffiniPure Donkey Anti-Rabbit (Jackson ImmunoResearch) secondary antibody was also used. Chemiluminescent signal was produced using Immun-Star AP substrate pack (BioRad Laboratories) and luminescence was detected with scientific imaging film (Kodak).

\textit{Image Analysis and Quantification}

Gel images were digitized at 200-300 dpi using a Epson Expression 10000XL device (Epson Electronics America, CA, USA). Protein spots were then quantified and matched with other gels using Progenesis Workstation 2005 software (NonlinearDynamics, New
Castle-upon-Tyne, UK). Composite images of each treatment group were normalized by means of a total spot intensity comparison between sample groups. Quantified spot changes were within the linear range of silver stain. The same platform was used to acquire and analyze densitometry data generated through western blotting analysis. In this case, the luminescent signal generated by each sample band and recorded on the imaging film was normalized against the density of the correspondent lane stained with the general protein staining DB71.

**Protein identification by Mass Spectrometry**

Protein spots were excised from fresh gels, and destained according to a modified protocol of Gharahdaghi et al. 

Proteins were digested in 25 mmol/L ammonium bicarbonate, pH 8.0 completed with 10 μg/mL sequencing grade modified porcine trypsin (Promega), for 16-24 h at 37°C. Peptides were extracted twice with 50 μL of acetonitrile (ACN) and 25 mmol/L ammonium bicarbonate 1:1 v/v for 60 min and then dried under vacuum. Tryptic peptides were reconstituted in 3 μL of 50% ACN/0.1% TFA and analyzed by electrospray ionization (ESI) MS/MS LCQ Deca XP Plus mass spectrometer (ThermoFinnigan, San Jose, California), as described previously.

Data-dependent acquisition was used to obtain both a survey spectrum along with several MS/MS spectra for multiply charged precursor ions present in each sample. MS/MS spectra were processed by baseline subtraction, and de-convoluted using Mascot wizard. Database searching was performed using Mascot wizard (www.matrixscience.com) using the “other-mammalian” sub-database of NCBInr protein databases. FASTA sequences were blasted against Swissprot protein database through the proteomics tool Expasy Blast.
(http://www.expasy.ch/tools/blast/) to further reduce protein redundancy. The number of unique peptides assigned by Mascot search and retrieval system is also listed for each protein. The Mowse score\(^9\) provided by the software was manually recalculated (Corrected Mowse) summing unique peptides as defined in\(^10\). Observed and theoretical isoelectric point (pI) and molecular weight (MW) values for identified proteins are given; bold font is used for observed values that differ significantly from the theoretical ones (\(\Delta pI \geq 1\) pH unit; \(\Delta MW \geq 10\) kDa). These parameters were used to assign protein identities when ambiguous IDs were retrieved by Mascot.

*Phosphopeptide Analysis by Immobilized Metal Affinity Chromatography (IMAC)*

Phosphopeptides were eluted to a C18 pre-column and analyzed by nanoflow HPLC/micro-ESI MS/MS via data-dependent analysis on an LCQ Deca XP Plus mass spectrometer (ThermoFinnigan, San Jose, California). Peptide identification was performed by searching the raw MS/MS spectra against the ATP\(\beta\) sequence (NCBI gi|73968432) using Bioworks 3.1 (ThermoFinnigan).

*ATP\(\beta\) De-phosphorylation Assay*

Mitochondrial pellets were re-suspended in 1% (w/v) SDS and 5% (w/v) ASB14 completed with protease inhibitor cocktail Complete \(^\text{TM}\), and then pooled in three groups: DHF pool, CRT pool and DHF+CRT pool, each mixed with equal protein quantities. The latter pool (alkaline phosphatase or AP pool) was then treated with alkaline phosphatase (CIP, New England Biolabs) overnight at 37°C. On the following day the pool samples were solubilized in CHAPS buffer and labelled with CyDyes (Cy3, Cy5 and Cy2 respectively)
for 20 minutes at room temperature. The labelling reaction was stopped by adding 20 mM Lysine to the samples. Samples were flash frozen or diluted in IEF buffer for two-dimensional electrophoresis. DHF and CRT pools were alternatively labelled with either Cy3 and Cy5 (dye swapping) to prevent artefact variations due to dye bias.

Blue Native PAGE (BN-PAGE)

Blue Native gel electrophoresis was performed using pre-cast native gels (Invitrogen) according to manufacturer’s instructions. Briefly, mitochondrial suspensions were thawed, centrifuged to remove the H-buffer, and diluted in blue native sample buffer completed with 2% Lauryl Maltoside, and incubated 30 min in ice. Samples were then centrifuged (20000 rcf, 30 min, 4°C) to remove insoluble material. Supernatants were completed with 5% Colloidal Coomassie Blue G-250 (0.25% final concentration) and separated for 1 hr 30 min at 150 V. Protein complexes were subsequently blotted onto PVDF as described in immunoblotting section.

In-gel ATPase Activity

The procedure described in previous section (BN-PAGE) was also followed to separate mitochondrial complexes for further determination of complex V activity as described by Bisetto et al. Briefly, BN-PAGE gels were equilibrated for two hours in equilibration buffer (33 mM Tris, 285 mM Glycine, 13 mM MgSO4). After equilibration the gels were incubated in fresh equilibration buffer completed with 8.8 mM ATP and 0.2% w/v Pb(NO3)_2, for 2 hours at room temperature. In these conditions the ATP-ase activity of F₁/F₀ hydrolyses ATP to ADP and inorganic phosphate. Phosphate forms a white insoluble
lead salt that precipitates locally and originates a white band whose density is proportional to ATP-synthase activity on the separated $F_1/F_0$ bands. Densitometric analysis was performed using a dark background screen and the values were normalized vs. the coomassie G250 signal of complex V band after separation.

**Measurement of Oxygen Consumption in Isolated Mitochondria**

Mitochondria were isolated from the left ventricles of sham, DHF and CRT dogs. Ventricles were placed in ice-cold isolation buffer (containing 225 mM mannitol, 75 mM sucrose, 1 mM EGTA, 20 mM Hepes, 0.2% BSA, pH 7.4), then rapidly minced and homogenized using the Polytron homogenizer at low speed. The homogenate was centrifuged at 500 g for 10 minutes at 4°C (Sorvall, SS34); the supernatant was filtered through a 150 μm mesh and centrifuged again at 8000 g for 10 minutes. The pellet was resuspended in isolation buffer without BSA, centrifuged at 8000 g for 10 min and the final pellet resuspended in a small volume of isolation buffer and stored on ice until analysis. Protein concentration was determined by bicinchoninic acid (BCA) protein assay (Pierce) using bovine serum albumine as a standard. Mitochondria (0.4 mg protein/ml) were suspended in standard medium containing 250 mM sucrose, 1 mM KH$_2$PO$_4$, 20 μM EGTA, 10 mM MOPS, pH 7.4, and 5/2.5 mM glutamate/malate or 5 mM succinate (in the presence of 2 μM rotenone) as substrates. Oxygen consumption was determined fluorometrically, using a fiber optic oxygen sensor (Ocean Optics, Inc). Assays were performed at 37°C, on instruments equipped with thermostatic control and magnetic stirring.

**Measurement of Isocitrate Dehydrogenase (IDH) Activity**
Mitochondrial matrix was prepared from freshly isolated mitochondria by sonication (3 bursts for 10 seconds) and ultracentrifugation (43 000 rpm for 30 min at 4°C). NADP\(^+\) dependent IDH activity was measured spectrophotometrically, following the reduction of NADP\(^+\) at 340 nm and 37°C in a buffer containing 33 mM KH\(_2\)PO\(_4\), 1 mM CN\(^-\), 1 mM MgCl\(_2\), 1 mM NADP\(^+\), pH 7.4. The reaction was initiated by the addition of 20 mM isocitrate. One unit of activity is defined as the production of 1 μmol NADPH min\(^{-1}\).

Protein concentration was determined by bicinchoninic acid (BCA) protein assay (Pierce), using BSA as a standard.

Statistical Analysis and Spot Selection Criteria

To address the issue of multiple comparisons and spurious associations, we considered controlling the family-wise error rate using the method of Bonferroni (dividing the type I error rate to declare statistical significance by the number of tests performed). However, Bonferroni correction was recently described to be “unduly strict” for this type of studies\(^{12}\). Therefore we also derived the q-values\(^{13}\) from the calculated p-values, which allows for statistical inference with emphasis on the false discovery rate (that is, we can quantify the expected proportion of false positives among the spots declared significant). Statistically significant protein changes were determined using two-tailed student’s t-tests of the normalized spot intensities between groups using a p value of less than 0.05. While none of the proteins spots reached statistical significance after a Bonferroni correction, the overall distribution of the p-values showed a very strong departure from a Uniform(0,1) distribution, which would have been expected under a global null (that is, no changes are observed in any of the proteins, Supplementary Figure 8).
discovery rate at 20%, a total of 76 spots are declared significant (of which we would expect approximately 15 to be false positives). Controlling the false discovery rate at a more stringent 10% still yields 28 significant spots (of which about 3 would be expected false positive). Thus, there is a large abundance of proteins that exhibit changes in concentration. The spots list, originally composed of over a hundred observations, was manually assessed to check for: consistency throughout the gels, signal saturation, MS identification susceptibility and mitochondrial localization, and therefore reduced to the final list presented in this study. Importantly, only protein changes $\geq 1.5$-folds were considered to account for biological significance, after performing statistical analysis. Some of the changes (around one-third, $q \geq 0.2$) would not normally be considered signals but were retained because of the support of other biologically related changes (e.g. subunits of the same complex changing coherently or co-ordinated changes in known metabolic pathways). Few of them may require further validation and q-values are reported for clarity in Table 2. Indeed, none of these proteins (individually) reaches the statistical significance required after Bonferroni correction. This, obviously, is a consequence of the very small sample size, and the fact that about 1200 proteins were observed on the gels and had to be corrected for when attempting to control the family wise error rate (which is not very desirable in such a setting, as the p-values for true and false positives overlap substantially, due to the small sample size). Note that none of these considerations explicitly address the magnitude of the observed differences.
Supplemental Results

Differential Display Analysis

Table 2 lists protein changes in alphabetic order. The abundance of 31 protein species was changed in CRT group compared with DHF. MS identification, based on MS/MS, is listed. In a few cases, more than one protein was identified in a single gel spot, in most of these cases, carryover was ruled out based on spectra quality, number of unique peptides, and pI/MW values. In two cases it was not possible to unambiguously assign protein identity based on aforementioned criteria (eg. SAM50-like protein/ATP-synthase alpha subunit and NADH-dehydrogenase Fe-S 30 kDa/Clp protease) therefore multiple IDs are maintained in Figure 2H). Fold changes are presented with a positive or negative sign to indicate when their level were respectively increased or reduced upon CRT. Mitochondrial proteins are numbered, whereas mitochondria-associated proteins are marked with letters.

Other Protein Changes

The SAM50-like protein CGI-51 was detected together with ATPα in a protein spot that increased with CRT (3-fold, p=0.02,q=0.24). Although we could not unambiguously assign the protein spot identity, it is likely that ATPα, being a very highly abundant protein in mitochondria, is a carryover contaminant. Moreover the experimental pI and MW values are compatible with the assignment to SAM50 since ATPα theoretical pI is considerably different from the observed value.

Another spot is decreased in CRT (-2-fold, p0.031, q=0.27) and contains both the casein-like ATP-activated protease ClpP and the 30 kDa Fe-S subunit of complex I NADH-
dehydrogenase. Subsequent immunoblotting analysis confirmed a reduction in ClpP levels (see online Supplementary Figure 5 and Figure 2H in the main text).

Additionally, two proteins that are known to associate with mitochondria were found to be modulated by CRT were significantly altered upon CRT (Figure 2I of main text), Desmin, an intermediate filament protein that is known to interact with and influence mitochondrial function\textsuperscript{14,15}, and GAPDH which interact with proteins on the cytosolic side of the outer mitochondrial membrane were increased in CRT (2- fold, p=0.003 and 0.02, and q=0.10 and 0.23 respectively).

\textit{ATPδ Subunit is Phosphorylated with CRT and DHF}

Another subunit of complex V, ATP-synthase δ (ATPδ), displayed a change in pI upon treatment with alkaline phosphatase as shown in Supplementary Figure 1C of online supplement. The dominant blue colour (Cy2) of the basic spot indicates an increase in the density of the spot, corresponding to the un-phosphorylated form of ATPδ upon AP treatment. Phosphorylation of ATPδ was also confirmed through two-dimensional western blotting analysis using a specific antibody (anti-ATP5D, Atlas Antibody- Sigma). Supplementary Figure 1D shows a representative 2D western blotting of DHF, CRT and AP.

\textit{Ingenuity Pathways Analysis}

FASTA sequences obtained from NCBI protein database listed in Table 2 were blasted against Uniprot database and the top ranking sequences were used to compile a dataset.
This also included the fold changes listed in Table 2. The dataset was uploaded into IPA and a core analysis was performed. The canonical pathways that were modulated significantly according to the algorithm along with other major canonical pathways are displayed in Supplementary Figure 3.

**Supplemental Discussion**

Intriguingly, a membrane protein of unknown function, XP_53391 is strongly reduced in the CRT group. The biological role of this protein in the pathophysiology of HF has not yet been characterized suggesting a valuable new series of experiments to define its role. The C-terminus part of XP_53391 protein is homologous to the same portion of zebra fish isocitrate dehydrogenase gamma isoform (gi|62955395). IDH activity was tested, and found to have increased with CRT (Supplementary Figure 4). The XP_53391 protein spot decreased with CRT. Protein modification (see pI shift in Table 2) or different IDH protein isoform expression may explain this reverse correlation. However, further investigations are needed to assign IDH activity to XP 53391.

SAM50-like protein CGI-51 (sorting and assembly machinery component 50 homolog) was detected together with ATPα, in a spot whose quantity was increased by 3-fold in CRT. SAM50-like protein is poorly characterized and was found in the outer mitochondrial membrane of human heart mitochondria in association with the mitochondrial protein import machinery. This evidence points to a potential increase in mitochondrial protein import capacity with CRT, in accordance with the observed reduction in protein turnover and improvement in mitochondrial protein synthesis.
We recently described a role for reduced apoptosis in the canine DHF model\textsuperscript{3}. ROS production has been pointed as one of the possible causes for contractile deficiency and heart failure also based on their capacity to activate the mitochondrial apoptotic pathway\textsuperscript{17, 18}. The remarkable increase in thioredoxin-dependent peroxide-reductase, found in the present study, suggests that the mitochondria in the CRT group are more protected against ROS production. Interestingly, another mitochondrial protein involved in redox protection and apoptosis regulation, programmed cell death 8 or apoptosis inducing factor (AIF), was elevated in the mitochondria obtained from CRT compared to DHF dogs. The role of AIF in oxidative stress protection and apoptosis is controversial\textsuperscript{19}. However, increased susceptibility to oxidative stress and increased apoptosis after ischemia/reperfusion injury and aortic banding were reported in AIF-deficient mouse mutants (Harlequin, Hq) compared to wild type, confirming the function of AIF as a ROS scavenger in the heart, in vivo\textsuperscript{20}. The contemporary increase in AIF levels observed in the present study, together with the reported reduced apoptosis in the same animal model, after CRT\textsuperscript{3}, support the hypothesis that AIF primary function in vivo is protection from ROS.

Finally, only few mitochondria-associated proteins were modified by CRT. The most important of these is desmin, that is the specific intermediate filament cytoskeleton protein in the cardiac myocyte and has shown the ability to modulate mitochondrial activity and localization\textsuperscript{14, 15}. Mitochondrial localization is crucial in ensuring proper energy production and delivery to sites with high energy demand. Cytoskeletal and myofibrillar disarrangement in cardiac myocytes is one of the early features that define cardiac failure;
we believe that intermediate filaments play a crucial role in regulating the maladaptive transition to the diseased phenotype, possibly mediated by mitochondria re-distribution. It is therefore not surprising that this protein is changed when bi-ventricular pacing is applied.

**Supplemental Figure Legends**

**Supplementary Figure 1.** Succinate dehydrogenase and ATP-synthase δ subunit phosphorylation. Two representative images of de-phosphorylation 2DE zoomed at succinate dehydrogenase are shown for ease of comprehension: with three Cy Dyes is shown in panel A (DHF in green (Cy3), CRT in red (Cy5) and the alkaline phosphatase-terminated pool (AP) in blue (Cy2)); and the classical two-dyes de-phosphorylation assay described by Raggiaschi et al., in panel B. In panel C is presented the doublet obtained de-phosphorylation of ATPδ. Cy-dye sample and color-assignment are provided. In panel D a two-dimensional western-blotting analysis using a specific anti-ATPδ is also presented confirming pI shift upon treatment with AP. Note the shift in pI upon treatment with alkaline-phosphatase (AP). ☺; positive control for second dimension (SDS-PAGE).

**Supplementary Figure 2.** ATPβ degradation and MS/MS sequence coverage. Protein spots identified as ATPβ are indicated by arrows and accompanied by serial spot numbers (SSN) in Panel A. Panel B shows the sequence coverage obtained during the MS/MS analysis for each individual spot. The sequence of the precursor protein (NCBI gi|73968432) is utilized, and the mitochondrial transit peptide adapted from the human sequence (47 aa, uniprot/swissprot database) is shown, together with its expected molecular weight. Bold red fonts indicate sequences covered by observed peptides. Red boxes
indicate the portion of the protein that is absent (i.e. not covered) in the specific protein fragment.

**Supplementary Figure 3.** Canonical pathways analysis through the Ingenuity Pathways Analysis (IPA) software. The ratio between the levels of protein in the CRT vs. DHF comparison are plotted along with a trend line (threshold) that divides the pathways that are significantly altered with CRT.

**Supplementary Figure 4.** Isocitrate activity in CRT and DHF hearts.

**Supplementary Figure 5.** Two-dimensional immunoblotting analysis for ClpP. The basic edge of 4-7 18 cm strip was cut and mounted on a small precast gel and subsequently blotted on PVDF. Anti-ClpP antibody (Sigma, Prestige Antibody, 1:2500 diluted) was used to probe the membrane. DHF, Dyssynchrony-induced Heart Failure; CRT, Cardiac Resynchronization Therapy; AP, alkaline phosphatase treatment.

**Supplementary Figure 6.** Experimental design and proteomics workflow. See main text for description. LBBB, left bundle branch blockade; TP, tachy-pacing; 2DE, two-dimensional electrophoresis; DHF, Dyssynchronous Heart Failure; CRT, Cardiac Resynchronization Therapy; AP, Alkaline Phosphatase; MS/MS, tandem mass spectrometry; IMAC, immobilized metal affinity chromatography.

**Supplementary Figure 7.** Mitochondrial enrichment protocol. Mitochondrial protein enrichment was evaluated by probing the fractions collected along with the enrichment protocol with specific antibodies for mitochondria (ATPβ) and myofilaments (Troponin I, TnI).

**Supplementary Figure 8.** Histogram of the protein p-values (A), and q-values for p<0.05 (B). The histogram shows a clear enrichment of low p-values, i.e. a strong departure from
a Uniform(0,1) distribution, which would be expected if no signal in the data existed. Q-values were calculated to address the multiple comparisons problem. The p-value cut-off for false discovery rate control at 10%, 20% and 30% were 0.003, 0.014, and 0.043, respectively. For example, declaring all differences with p<0.013 significant yields 62 spots, of which about 12-13 would be expected false positives.
Supplementary Figure 1

A. DHF~Cy3, CRT~Cy5, AP~Cy2

B. DHF~Cy3, AP~Cy2

C. ATP delta synthase

D. 17 kDa-
   DHF~Cy3 CRT~Cy5 AP~Cy2

≈4.25 pl ≈4.50
## Supplementary Figure 2

### Mascot Search Results

#### Protein View

**Match to:** gq|719068432 Score: 1526

**Predicted:** similar to ATP synthase beta chain, mitochondrial precursor isoform 1 (Canis familiaris)

**Found in search of:** C:\\Users\\[username]\\Desktop\\[project name]\\venv\\venv\\py36\\canis_familiaris_blast

**Nominal mass:** Mw, kDa

<table>
<thead>
<tr>
<th>MW, kDa</th>
<th>200</th>
<th>116.3</th>
<th>97.4</th>
<th>66.3</th>
<th>55.4</th>
<th>36.5</th>
<th>31.0</th>
<th>21.5</th>
<th>14.4</th>
<th>6</th>
</tr>
</thead>
</table>

**pI:** 4

**Fixed modifications:** Carbamidomethyl (K)

**Variable modifications:** Oxidation (M)

**Cleavage by:** Trypsin: cuts C-terminal side of KE unless last residue in P

**Sequence Coverage:** 38%

**Matched peptides shown in Bold Red**

### Transit peptide in human sequence

**UniProtKB/Swiss-Prot P06576 (ATPB_HUMAN), ~5KDa**

---

**A.**

**ATP synthase β subunit**

**B.**

**Intact, unphosphorylated**

**Supplementary Figure 2**

**UniProtKB/Swiss-Prot P06576 (ATPB_HUMAN), ~5KDa**

**Transit peptide in human sequence**

**UniProtKB/Swiss-Prot P06576 (ATPB_HUMAN), ~5KDa**
Supplementary Figure 3
Supplementary Figure 4

Isocitrate dehydrogenase activity

$p<0.05$
Supplementary Figure 5

pH ≈ 5.5  pH ≈ 6.5

28 kDa

DHF

28 kDa

CRT

28 kDa

AP
**Supplementary Figure 6**

LBBB+TP

\[ \downarrow \quad 6 \text{ wks} \]

Dyssynchrony-induced Heart Failure (**DHF**)  

LBBB+TP \quad CRT+TP

\[ \downarrow \quad 3 \text{ wks} \quad \downarrow \quad 3 \text{ wks} \]

Cardiac Resynchronyization Therapy (**CRT**)  

Mitochondria enriched fractions

\[ \downarrow \]

2DE, differential display analysis

\[ \downarrow \]

MS/MS protein identification

\[ \downarrow \]

IMAC phospho-peptide enrichment

\[ \downarrow \]

Mitochondria functional characterization

\[ \downarrow \]

In-gel de-phosphorylation assay

\[ \downarrow \]

PO$_4^2$  

Fe$^{3+}$

**DHF~Cy5**  

**CRT~Cy3**  

**AP~Cy2**

**merged**
Supplementary Figure 7

- **Cell debris, nuclei, myofilaments**
- **Crude mitochondria**
- **Mitochondria/myofilament**

**Mitochondrial proteins enrichment**

**MW, kDa**
- 200
- 116.3
- 97.4
- 66.3
- 55.4
- 36.5
- 31.0
- 21.5
- 14.4
- 6.0
- 3.5

**Differential centrifugation**
- Total extract
- Cell debris, nuclei, myofilaments
- Loss in washing/concentrating
- Crude mitochondria
- Mitochondrial/myofilament
- Enriched mitochondria

**ATPβ (mitochondria)**

**Tnl (myofilaments)**
Supplemental References


16. Xie J, Marusich MF, Souda P, Whitelegge J, Capaldi RA. The mitochondrial inner membrane protein mitofilin exists as a complex with SAM50, metaxins 1 and 2, coiled-


