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Mice Over-Expressing the Myocardial Creatine Transporter Develop Progressive Heart Failure and Show Decreased Glycolytic Capacity

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Abstract

The metabolic phenotype of the failing heart includes a decrease in phosphocreatine and total creatine concentration [Cr], potentially contributing to contractile dysfunction. Surprisingly, in 32 week old mice over-expressing the myocardial creatine transporter (CrT-OE), we previously demonstrated that elevated [Cr] correlates with left ventricular (LV) hypertrophy and failure. The aim of this study was to determine the temporal relationship between elevated [Cr] and the onset of cardiac dysfunction and to screen for potential molecular mechanisms. CrT-OE mice were compared with wild-type (WT) littermate controls longitudinally using cine-MRI to measure cardiac function and single-voxel ¹H-MRS to measure [Cr] *in vivo* at 6, 16, 32, and 52 weeks of age. CrT-OE mice had elevated [Cr] at 6 weeks (mean 1.9-fold), which remained constant throughout life. Despite this increased [Cr], LV dysfunction was not apparent until 16 weeks and became more pronounced with age. Additionally, LV tissue from 12 to 14 week old CrT-OE mice was compared to WT using 2D difference in-gel electrophoresis (DIGE). These analyses detected a majority of the heart's metabolic enzymes and identified 7 proteins that were differentially expressed between groups. The most pronounced protein changes were related to energy metabolism: α - and β -enolase were selectively decreased ($p < 0.05$), while the remaining enzymes of glycolysis were unchanged. Consistent with a decrease in enolase content, its activity was significantly lower in CrT-OE hearts (in WT, 0.59 ± 0.02 μ mol ATP produced/ μ g protein/min; CrT-OE, 0.31 ± 0.06 ; $p < 0.01$). Additionally, anaerobic lactate production was decreased in CrT-OE mice (in WT, 102 ± 3 μ mol/g wet myocardium; CrT-OE, 78 ± 13 ; $p = 0.02$), consistent with decreased glycolytic capacity. Finally, we found that enolase may be regulated by increased expression of the β -enolase repressor transcription factor, which was significantly

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increased in CrT-OE hearts. This study demonstrates that chronically increased myocardial [Cr] in the CrT-OE model leads to the development of progressive hypertrophy and heart failure, which may be mediated by a compromise in glycolytic capacity at the level of enolase.

Keywords

Myocardial energy metabolism; heart failure; hypertrophy; creatine content; magnetic resonance imaging; magnetic resonance spectroscopy; proteomics

Introduction

A large body of work suggests that impaired myocardial energetics may be one factor contributing to the patho-physiology of contractile dysfunction in heart failure [1–8]. This is supported by the observation that energy-sparing therapeutics are beneficial in heart failure [5,9–11], while energy-costly drugs worsen prognosis [2,5,12,13]. A hallmark of the metabolic profile in heart failure, observed in both humans and animal models, is substantially reduced levels of myocardial phosphocreatine (PCr) and a loss of the total creatine (Cr) pool [14–20]. Transgenic mouse models of impaired cardiac energetics have added insight into the role of myocardial high-energy phosphate metabolism [21]. Impaired contractile reserve has been observed in creatine kinase [22] and guanidinoacetate-N-methyltransferase [23] knock-out mice, as two models of impaired myocardial phosphocreatine availability and content, respectively. Most recently, we followed the reverse strategy and created a mouse model with excess levels of myocardial PCr and Cr, by transgenically over-expressing the myocardial creatine transporter (CrT-OE) [24]. Our original study in this model showed a large variation in Cr accumulation, with up to 4-fold increase in myocardial Cr content [24]. Initially hypothesized to serve a protective role, animals with Cr levels >2-fold of normal developed left ventricular hypertrophy and heart failure [24]. This study speculated that such hearts failed because they were unable to keep the increased Cr-pool adequately phosphorylated as PCr, resulting in augmented free ADP levels and reduced free energy available from ATP hydrolysis [24], but it did not define these mechanisms further.

To further understand how the heart is affected by increased myocardial Cr concentrations, we asked the following questions: What is the time course of Cr accumulation in CrT-OE hearts over the first year of life? Is creatine accumulation strictly associated with cardiac functional changes, or can it be dissociated at young age? And, finally, what are the molecular changes associated with hypertrophy and heart failure in this model? To answer these questions, we used *in vivo* MR imaging and spectroscopy to sequentially monitor cardiac function and creatine levels over one year, and proteomics to screen for differences in myocardial proteins. Our study demonstrates that myocardial [Cr] elevation is constant over the first year of life, and that progressive LV dysfunction is initiated between 6 and 16 weeks of age. Additionally, this work identified 7 proteins that were differentially expressed between WT and CrT-OE hearts. Strikingly, enolase was selectively decreased in CrT-OE hearts. Enolase is a glycolytic enzyme that catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate, a high-energy intermediate that produces ATP through distal glycolysis. Consistent with a down-regulation in enolase content, its activity as well as anaerobic lactate production were significantly decreased in CrT-OE mice. Furthermore, CrT-OE hearts showed increased expression of the β -enolase repressor factor, suggesting that enolase may be regulated at the transcriptional-level in this model. Thus, the current study provides insight into the potential mechanisms leading to progressive heart failure in CrT-OE mice.

Materials and Methods

CrT-OE Mice

CrT-OE mice were generated by mating male transgenics with female C57Bl/6 mice. These mice were genotyped by PCR as previously described [24]. Mice were housed on a 12 hour light/dark cycle at $21\pm 2^{\circ}\text{C}$ and provided free access to Cr-free chow and water ad libitum. All animal procedures were approved by the University of Oxford Animal Ethics Review Committees and the Home Office (London, UK).

In Vivo Cine-MRI and ^1H -MRS in CrT-OE Mice

Forty mice, 20 from each sex (i.e., 13 CrT-OE and 7 WT), were used for longitudinal imaging at 6, 16, 32, and 52 weeks. Mice were prepared for MR-experiments as previously described [25]. Briefly, mice were anesthetized with 2% isoflurane in 100% O_2 administered via a nose cone and placed supine within a custom-built, heated cradle. Cardiac and respiratory signals were constantly monitored using an in-house-developed ECG and respiratory gating device [26]. Experiments were performed in a vertical-bore, 11.7-T (500-MHz) MR system (MagneX Scientific, Oxon, UK) with a Bruker Avance console (Bruker Medical, Ettlingen, Germany) [23] (Figure 2.2B). Quadrature driven birdcage coils with inner diameters of 28 and 40 mm (Rapid Biomedical, Würzburg, Germany) were used to transmit/receive the nuclear magnetic resonance (NMR) signals.

High resolution cine-magnetic resonance imaging (cine-MRI) was performed *in vivo*, at all four time-points, to assess left ventricular (LV) mass and volumes, as previously described [25,27]. Briefly, 8 to 10 contiguous slices were acquired in short-axis orientation, covering the heart from base to apex (spatial resolution, $100\ \mu\text{m} \times 100\ \mu\text{m} \times 1\ \text{mm}$; temporal resolution, 4.6 msec), with a cardiac-triggered and respiratory-gated fast low-angle-shot sequence. Between 18 and 32 frames per heart cycle and 2 to 16 phase encoding steps per respiration cycle were acquired in a segmented fashion for each animal. Cine-MRI data were processed off-line with purpose-written-IDL (Research Systems International, Crowthorne, Berkshire, UK) and AmiraTM 2.3 software (TGS Europe, Mérégnac Cedex, France) as previously described [27], to determine LV mass, end-diastolic volume (EDV), end-systolic volume (ESV), LV ejection fraction (LVEF), stroke volume (SV), and cardiac output (CO).

Immediately following cine-MRI, *in vivo* ^1H -MRS was performed as previously described [28]. In brief, cardiac-triggered and respiratory-gated, water suppressed and unsuppressed, cardiac spectra from a $2\ \mu\text{l}$ voxel, positioned in the inter-ventricular septum, were acquired in diastole using a PRESS sequence (TE=9 msec, TR $\approx$ 2 sec, repeated twice). Water-suppressed spectra, consisting of 256 averages, were acquired on-resonance on Cr. ^1H -MRS data were analyzed with a time-domain fitting software, jMRUI [29], with the Cr signal referenced to the unsuppressed water signal. After the final imaging time-point, animals were euthanized and the LV was excised, blotted, weighed, and frozen in liquid nitrogen for subsequent analyses. Myocardial Cr content was converted from the relative units of ^1H -MRS to absolute units (nmol Cr per mg protein) using a calibration curve of the ^1H -MRS and HPLC values obtained at the 52 week time point.

Tissue from aortic banded mice

Adult male C57BL/6 mice were subjected to transverse aortic constriction or sham surgery (n=6 of each), with echocardiography and tissue harvest after 5 weeks, as previously described in detail [30].

Chemicals and Buffers

All chemicals, including ATP, KCN, antimycin-A, and salts, were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise stated. All reagents and instruments used for 2D gel electrophoresis were purchased from GE Healthcare, formerly Amersham BioSciences (Piscataway, NJ), unless otherwise specified.

Left Ventricular Tissue Preparation for use in Proteomic and Biochemical Analyses

For proteomic and biochemical experiments, 12 to 14 week old female CrT-OE and WT siblings were used. Female mice were used to eliminate a gender effect when comparing the protein profiles of CrT-OE and WT hearts. The mean LV Cr contents for the animals used in these studies were 70 ± 8 nmol Cr per mg protein for WT and 142 ± 43 for CrT-OE. Directly following euthanasia, the LV was excised, rinsed 3-times in cold $1 \times$ phosphate-buffered saline (PBS) with heparin, blotted, and frozen in liquid nitrogen. Frozen tissue was subsequently powdered in a stainless steel percussion mortar cooled in liquid nitrogen. [31][32][32][31][31]

2D Gel Electrophoresis, Gel Staining, Image Acquisition, and Protein Identification

Powdered LV tissue was solubilized in lysis buffer (15 mM Tris-HCl, 7 M urea, 2 M thiourea, and 4% CHAPS (w/v)) by vortexing and sonicating on ice. The crude extract was centrifuged at 100,000 g for 30 min at 15°C, and the supernatant was assayed for total protein concentration using the USB Quant kit (USB Corp., Cleveland, OH).

To screen for protein differences and obtain quantitative protein ratios, 2D difference gel electrophoresis (DIGE) was performed on 4 paired samples (4 CrT-OE and 4 WT), as previously described [33]. Briefly, 50 µg of WT and 50 µg of CrT-OE sample were labeled with Cy3 and Cy5 dyes, respectively. Twenty-five µg of each sample was mixed together and labeled with Cy2 to serve as an internal standard. CyDye labeling was quenched with 10 mM lysine for 15 min before combining. DIGE and unlabeled protein (500 µg/gel) samples were loaded onto 24 cm Immobiline DryStrip gels (pH 3–10NL) and isoelectric focusing (Ettan IPGphor2 apparatus) was achieved by active rehydration at 30 V for 12 hr, followed by stepwise application of 250 V, 500 V, 1000 V, and 8000 V for a total of ~72,000 Vhr. After focusing and equilibration, strips were applied to 10–15% SDS PAGE gels (Jule, Inc., Milford, CT) and sealed with 1% agarose, containing bromophenol blue. Electrophoresis was performed at 20°C in an Ettan DALT-12 tank with electrophoresis buffer (25mM Tris, pH 8.3, 192mM glycine, and 0.2% SDS) for a total of ~2000 Vhr. Gels were subsequently imaged using a Typhoon 9400 scanner at 100 µm resolution, with previously described imaging parameters [33]. Unlabeled gels for mass spectrometry identification were fixed for 4 hours in a solution of 30% methanol and 3% phosphoric acid, and then stained overnight with Sypro Ruby dye (Bio-Rad Laboratories).

A custom written image processing program was used to correct for scanning gain/detector alterations for DIGE images [33]. DeCyder 2D Differential Analysis Software was used to match proteins of interest identified in DIGE to Sypro-Ruby(Bio-Rad Laboratories, Hercules, CA) stained images [34], which were subsequently picked using an Ettan Spot Handling Workstation. Protein identification was carried out using a MALDI-TOF/TOF instrument (4700 Proteomics Analyzer, Applied Biosystems, Foster City, CA) with MS/MS peptide analysis. At least two peptides were obtained for each protein using MS/MS. The MS/MS spectra were searched against the National Center Biotechnology Information (NCBI) mouse protein database using the MASCOT search algorithm (Matrix Science, Boston, MA). A positive identification was indicated by >95% confidence in the MASCOT identification.

Immunoblotting

Western blot analysis was performed on an LV protein homogenates from 10 WT and 10 CrT-OE mice using standard methods with enhanced fluorescence. Briefly, 40 μ g of protein were run on a 12.5% SDS gel and transferred to a PVDF membrane. After incubating with Starting Block Solution (Pierce, Rockford, IL) for 30 min, membranes were probed for β -enolase repressor factor 1 (β ERF-1) (Santa Cruz Biotechnology, Santa Cruz, CA, sc-48811) and β -actin (AbCam, Cambridge, MA, ab8227).

Biochemical Measurements

Total Cr content was measured using HPLC, as previously described [18]. Cr content was expressed in nanomoles per milligram of protein (nmol Cr/mg protein). Enolase activity was measured according to a previously described bioluminescence method [35] using an ATP determination kit (Invitrogen, Carlsbad, CA), with slight modifications to the enzyme buffer. For this analysis powdered LV tissue from 7 CrT-OE, 6 WT, 6 aortic banded, and 6 sham control hearts was made up to a concentration of 10 mg/ml in a buffer containing 50 mM Tris-acetate, 1 mM EDTA, 1 mM EGTA, 3 mM potassium-acetate, 2 mM AMP, 2 mM DAPP, 400 units per ml of pyruvate kinase, 2 mM ADP, 5 mM potassium-phosphate, 5 mM MgCl_2 , 5 μ M antimycin A, 100 μ M KCN, 10 μ M oligomycin-B, and ATP monitoring reagents (ATP determination kit, Invitrogen, Carlsbad, CA). After obtaining a baseline measurement, 1 mM 2-phosphoglycerate was added and ATP production was measured over the course of 1 min. This assay takes advantage of coupled reactions in which ATP is produced as the final product by pyruvate kinase; under these reaction conditions the amount of ATP formed is directly proportional to enolase activity [35]. All assays were performed in quadruplicate.

Lactate production was measured in 5 CrT-OE mice and 4 WT siblings using a blood-gas analyzer (Ciba Corning 865, Ciba Corning Diagnostics, Medfield, MA) and a lactate biosensor (Cambridge Life Sciences, Cambridge, UK). To measure lactate production, powdered LV tissue was made up to a concentration of 30 μ g/ml in a buffer containing 125 mM KCl, 15 mM NaCl, 20 mM HEPES, 1 mM EGTA, 1 mM EDTA, 5 mM MgCl_2 , 100 μ M KCN, 5 μ M antimycin-A, 5 mM ATP, and 20 mM glucose. Samples were vortexed briefly and incubated for 30 min at 37°C. Perchloric acid was added to a final concentration of 6% and the sample was vortexed and deprotonized on ice for 5 min. Samples were centrifuged at 3000 g for 5 min at 4°C, and the supernatant was neutralized with 3 M Tris-HCl (pH 8.8). Samples remained on ice until analysis and were analyzed in random order. Six assays were performed for each sample.

Statistical analysis

For the MR-experiments, means and 95% confidence intervals of the cardiac parameters stratified genotype (CrT-OE vs. WT) were plotted at the four time design points (6 weeks, 16 weeks, 32 weeks and 52 weeks). Further exploratory analyses were conducted by examining the nonparametric curve estimates of the cardiac parameters versus time and genotype [36]. Results of these exploratory analyses were used to guide the choice of statistical models in the linear mixed effects model analysis. Statistical inferences for the longitudinal effects of genotype on the cardiac parameters over the 52 week time course were evaluated using the first and second order linear mixed effect models with diagonal and unstructured intra-subject correlation matrices, where differences were considered significant at $p < 0.05$. The Cox Proportional Hazard Models were used to assess the effects of genotype and other risk factors on the overall survival time with right censoring. The statistical significance between groups at each time point was assessed by Student's *t-test*, where differences were considered significant at $p < 0.05$. The 2D DIGE proteomics data was tested for statistical significance as previously described [34]. The β ERF-1 and anaerobic lactate results were assessed by Student's

t-test, with $p < 0.05$ considered significant. Pearson correlations were used to evaluate the statistical associations of multivariate baseline variables.

Results

Longitudinal Assessment of Cardiac Function and Metabolism

In general the MR protocol was well-tolerated by mice. No significant difference in mortality was observed; 3 CrT-OE and no WT mice died over the 52 week time-course of this study. In all cases, mice either failed to recover from general anesthesia or died a few days after the 32 week MR-exam.

Creatine content—*In vivo* ^1H -MRS revealed that CrT-OE mice had significantly increased myocardial Cr levels averaging 1.9-fold above normal over 52 weeks. Representative spectra from WT and CrT-OE hearts as well as mean Cr contents for each time-point are shown in Figure 1. As previously described [24], CrT-OE hearts showed considerable variability in myocardial Cr accumulation, with values ranging from 70 to 235 nmol per mg protein, and a mean of 131. ^1H -MRS was successful in 279 out of 312 cases (89%).

Cardiac function and mass—A comparison of the cardiac parameters for WT and CrT-OE mice at all four time points are presented in Table 1. Body weight increased as expected in both groups with no significant differences between WT and CrT-OE mice. For the first 32 weeks of the study, LV mass was similar in WT and CrT-OE hearts; however, significant LV hypertrophy developed by the 52 week time-point. This was confirmed by subsequent autopsy, where at 52 weeks, the mean LV mass of CrT-OE mice was 148 ± 24 mg, compared to 118 ± 20 mg for WT ($p < 0.01$).

All heart rate measurements were similar between groups and time-points. Cine-MRI revealed that LV ejection fraction was normal in both WT and CrT-OE hearts at the early time point of 6 weeks (Table 1 and Figure 2). Thus, despite increased Cr content at 6 weeks of age, CrT-OE mice showed normal cardiac function at this early time-point. While cardiac function remained normal in WT mice over 52 weeks, from 16 weeks onwards, CrT-OE mice showed significant and progressive impairment of LVEF compared with WT mice (Table 1 and Figure 2B). This impairment was associated with increased in LVESV, which was significantly larger in CrT-OE mice and increased progressively after 16 weeks, with little change in LVEDV over 52 week protocol (Table 1). After 32 weeks of age, stroke volume and cardiac output were also significantly reduced in CrT-OE hearts. As shown in Figure 3A, the mean Cr concentration over the study period correlated closely with LV ejection fraction at 52 weeks. Furthermore, Figure 3B shows that the myocardial Cr level at 6 weeks predicted the loss of ejection fraction occurring over the subsequent 46 weeks.

Thus, the current study demonstrates that while CrT-OE mice have normal cardiac function early in life, their increased myocardial Cr levels predicted the loss of cardiac function over 52 weeks. This finding implies that increased Cr content contributed, either directly or indirectly, to the progressive development of heart failure and LV hypertrophy in the CrT-OE model.

CrT-OE Proteomics Analysis

Since our initial study [24] and the present longitudinal MR-experiment demonstrated a correlation between increased myocardial Cr content and the development of heart failure, we next sought to assess whether any direct biochemical changes could be observed at the protein level. A 2D difference in-gel electrophoresis (DIGE) analysis of WT and CrT-OE hearts identified 223 proteins from several biochemical pathways, including glycolysis, Krebs cycle,

fatty acid oxidation, and ketone synthesis. Across all analyses, the 2D DIGE approaches revealed 7 proteins with expression changes of at least 1.3-fold between WT and CrT-OE hearts (Figure 4A and Table 2). Among these proteins, the β -myosin heavy chain was increased in CrT-OE mice, consistent with previously reported hypertrophy [24]. Additionally, α - and β -enolase were down-regulated in CrT-OE hearts. The decrease in enolase content was interesting, in that of the seven glycolytic enzymes detected in this study, enolase was the only one with differential expression levels between WT and CrT-OE hearts (Supplemental Figure 1). It is important to note that aside from the 7 proteins listed in Table 2, the 2D DIGE image shown in Figure 4A revealed several additional proteins that appeared to differentially expressed. However, these proteins were not found to be statistically significant across all analyses ($p < 0.05$), and were therefore excluded from this screen for molecular alterations in CrT-OE hearts.

Enolase Activity and Anaerobic Lactate Production Measurements

To determine the functional effects of decreased enolase content in CrT-OE hearts, enolase enzyme activity and anaerobic lactate production were measured in LV tissue extracts. Consistent with a decrease in enolase content, enzyme activity was significantly lower in CrT-OE hearts (in WT, 0.59 ± 0.02 $\mu\text{mol ATP produced}/\mu\text{g protein/min}$; CrT-OE, 0.31 ± 0.06 $p < 0.01$) (Figure 5A). Since the CrT-OE model develops hypertrophy—and previous reports have shown that enolase activity is decreased in the hypertrophied heart [37]—enzyme activity was also measured in age matched CrT-OE and aortic banded hearts to more precisely determine the relationship between enolase activity and increased myocardial CrT content. Aortic-banded mice had significant LV hypertrophy, with post-mortem LV weight 155 ± 33 mg versus 93 ± 20 mg for sham controls ($p = 0.003$), but did not have congestive heart failure since lung weights were not significantly different. Similar to previous work [37], we found enolase activity to be suppressed in the hypertrophied LV (in sham control, 0.45 ± 0.02 $\mu\text{mol ATP produced}/\mu\text{g protein/min}$; aortic banded model of hypertrophy, 0.58 ± 0.03 ; $p < 0.05$). Importantly, relative to their controls, the CrT-OE model showed a ~46% decrease in enolase activity compared to ~22% in the aortic banded model. There was a strong correlation between increased myocardial Cr content and decreased enolase activity (Figure 5B) implying that, while its effects are additive to those of LV hypertrophy, [Cr] is the mediator of enolase activity in CrT-OE hearts.

To determine whether the decrease in enolase activity observed for CrT-OE mice led to diminished glycolysis, we next measured anaerobic lactate production in LV tissue extracts. As shown in Figure 6A, CrT-OE hearts showed decreased lactate production (WT mean, 102 ± 3 $\mu\text{mol/g wet myocardium}$; CrT-OE mean, 78 ± 13 ; $p = 0.02$), which was closely correlated with myocardial Cr levels (Figure 6B). This ~31% decrease in lactate production suggests that glycolytic flux is impaired in the CrT-OE model at the level of enolase.

β -enolase Repressor Factor 1 Expression Levels

To the best of our knowledge, β -enolase is the only glycolytic enzyme known to be regulated by its own transcription factor: β -enolase repressor factor 1 (βERF1), which is a negative regulator of β -enolase gene transcription. To determine whether the decrease in enolase content in CrT-OE hearts was mediated transcriptionally, we screened for differences in βERF1 expression between WT and CrT-OE hearts. Given the relatively low abundance of transcription factors in the cell, we were unable to detect βERF1 using the 2D DIGE approach. However, immunoblotting revealed that CrT-OE hearts have increased expression levels of βERF1 with respect to WT controls (~82%, $p < 0.01$) (Figures 7A–C). Notably, the strong correlation between myocardial Cr content and βERF1 expression (Figure 7D) suggests that [Cr] may be the driving force for decreased enolase content in this model.

Discussion

This study sought to characterize the time course, extent and mechanism whereby increased myocardial Cr content leads to cardiac dysfunction. First, we demonstrated that ^1H -MRS can be used as a tool to non-invasively monitor myocardial metabolism, in this case myocardial Cr content, over one year. Second, we showed that Cr levels are elevated and constant in CrT-OE hearts over one year of age. Third, we demonstrated that the development of hypertrophy and heart failure was progressive in the CrT-OE model. That is, at 6 weeks of age, cardiac function and LV mass were normal in CrT-OE mice despite elevated Cr levels, with cardiac dysfunction developing and worsening between 6 and 52 weeks of age. Fourth, we showed that enolase content was selectively decreased in CrT-OE hearts, which resulted in suppressed enolase activity and impaired glycolytic capacity. Finally, we propose that increased expression of the β -enolase repressor factor 1 may lead to the down-regulation of enolase content in CrT-OE hearts, which may play a role in the development of heart failure and LV hypertrophy in this model.

The current study used a longitudinal MR protocol to investigate the temporal relationship between increased total intracellular Cr content and cardiac function and mass. We showed that an elevation in myocardial Cr levels of 2-fold was attained by 6 weeks of age and remained constant over the 52 week protocol. Our initial phenotypic study on CrT-OE mice suggested that the development of LV hypertrophy and heart failure were due to augmented free ADP levels and subsequent drop in the heart's free energy change levels, resulting from the heart's inability to keep the increased Cr-pool adequately phosphorylated as PCr [24]. However, the current study showed that, at the early 6 week time-point, function was still normal despite elevated total Cr content, with contractile dysfunction not developing until 16 weeks of age. One potential explanation for this may be that the phosphorylated fraction of Cr (i.e., PCr) initially keeps up with the increase in total Cr (i.e., PCr increases proportionally to total Cr), thus keeping free ADP levels normal, and only with progressive age do hearts become incapable of keeping the augmented Cr pool adequately phosphorylated. In other words, after 6 weeks of age, the phosphorylated Cr fraction drops and free ADP increases, leading to contractile dysfunction, as shown in our earlier work using isolated heart ^{31}P -MRS to measure PCr in ~32 week old mice [24]. In principle, this hypothesis could be tested by measuring PCr and total Cr contents in the 6 week old mice. Unfortunately, due to their miniature size, we were unable to obtain spectra with sufficient signal-to-noise ratio in 6 week old perfused mouse hearts that would allow us to detect a 20–30% change in PCr. With further technological development in MRS measurements this may ultimately become feasible. Also, in our experience, HPLC measurements of PCr are not accurate or stable enough to allow assessment of the anticipated difference in levels. However; here we demonstrate an additional explanation for the progressive development of heart failure related to substrate utilization.

Using proteomic approaches we screened for potential molecular changes leading to contractile dysfunction in CrT-OE mice. It is important to note that since contractile dysfunction in the CrT-OE model developed between 6 and 16 weeks of age, animals aged 12–14 weeks were used for the proteomic and biochemical analyses. We felt that this relatively young age provided the best opportunity to observe the potential molecular mechanisms contributing to heart failure and hypertrophy in CrT-OE mice, as opposed to the biochemical changes associated with heart failure. Although hundreds of cardiac proteins were detected in the 2D DIGE analysis, only 7 proteins were differentially expressed in WT and CrT-OE mice with more than a 1.3-fold change. The detection of relatively few protein differences in CrT-OE hearts was advantageous because it implied that increased myocardial Cr content resulted in rather specific modifications.

Among the most notable protein modifications in CrT-OE hearts involved the selective down-regulation of α - and β -enolase (Supplemental Figure 1). A decrease in enolase content has been reported in the diabetic heart [33,38] as well as models of cardiomyopathy [39] and hypertrophy [40,41]. In the diabetic heart several glycolytic enzymes were down-regulated [33] and in the hypertrophied heart a decrease in β -enolase was coupled to a compensatory increase in α -enolase [40] or phosphofructokinase-1 [41]. In contrast, α - and β -enolase were both decreased in CrT-OE hearts, with no changes to the additional six glycolytic enzymes detected. Since changes in metabolic flux typically result from alterations to an entire biochemical pathway, as opposed to single rate-limiting steps [42,43], it was surprising that the down-regulation of enolase alone could mediate a change in energy-metabolism. In this context, it is interesting that a metabolic myopathy caused by a specific deficiency of β -enolase (glycogenosis type XIII) has been identified [44]. The patient suffering with this disorder demonstrated a progressive decline in skeletal muscle function, eventually preventing performance of sustained muscle exercises [44]. In analogy, it is conceivable that a specific decrease in myocardial enolase content may be a contributor to progressive heart failure in CrT-OE mice. Consistent with the decrease in enolase activity, CrT-OE mice showed a significant suppression in enolase enzyme activity and anaerobic lactate production, which suggests reduction in glycolytic flux (Figures 5 and 6). Furthermore, strong correlations were shown between decreased enolase activity/lactate production and increased total Cr content, suggesting a direct link between increased myocardial Cr levels and impaired glycolytic capacity in the CrT-OE model. The contribution of glycolysis to the cell's overall ATP content is generally thought to be minor under "normal" conditions, making it unlikely that decreased glycolytic capacity alone could contribute to heart failure. However, Aasum et al determined that glucose utilization contributes more to the overall myocardial ATP production in the mouse than in other species [45]. While fatty acids are the preferred oxidative substrate in human and rat heart [46,47], glucose appears to be favored in mouse heart [45]. Therefore, glycolytically-derived ATP may be preferentially used for contraction in the mouse heart, potentially explaining why decreased glycolytic flux may be detrimental in CrT-OE mice. Collectively, these findings imply that to the development of progressive hypertrophy and heart failure may in part be mediated by a compromise in glycolytic capacity at the level of enolase. [48,49] [49]

There is evidence that β -enolase, through its interaction with other proteins, may play a role in intracellular energy transfer. Previous studies have observed *in vitro* binding of β -enolase to pyruvate kinase, phosphoglycerate mutase, muscle creatine kinase (MM-CK), aldolase and troponin [50,51]. Additionally, a weak *in vivo* interaction has been observed between β -enolase and MM-CK [52]. Since the location of this " β -enolase protein complex" is favored near the sarcomeric apparatus, down-regulation of β -enolase in CrT-OE hearts may cause contractile dysfunction by impairing delivery of glycolytically-derived ATP to the subcellular sites where energy is needed for contraction. Collectively, these data are consistent with the notion that decreased glycolytic capacity may lead to progressive heart failure in CrT-OE mice.

Metabolic fluxes can be regulated by altering enzyme concentration, post-translational modifications, and the interaction of enzymes with substrates, products or allosteric effectors. A recent study in yeast demonstrated that glycolytic flux is largely controlled by the regulation of protein synthesis and degradation [53]. Although little is known about the mechanisms of translational regulation of glycolytic proteins, the available reports emphasize the regulatory role of transcription factors [54]. Thus, we screened WT and CrT-OE hearts for changes in protein content of the β -enolase repressor factor-1 (β ERF-1), which is a negative regulator of β -enolase gene transcription. CrT-OE hearts showed more than an 80% increase in β ERF-1 content, with respect to WT controls (Figure 7), suggesting that expression of β -enolase in CrT-OE heart may be regulated at the level of transcription. Since little is known about the expression level of β ERF-1 and its activity, further work is required to interpret the biological

significance of this finding. In addition to the decrease in enolase content, it is also important to point out that the expanded panel in Figure 4B shows an isoelectric shift for both α - and β -enolase toward the acidic region of the gel, consistent with a post-translational modification such as phosphorylation, glycosylation or oxidation. Importantly, phosphorylation of enolase has previously been demonstrated [55], and more recently, hyper-phosphorylation of α -enolase was shown to decrease enzymatic activity in the hypertrophied LV [37]. While further experimentation is required to identify and determine whether enolase is post-translationally modified in CrT-OE hearts, it is important to point out that decreased enolase content may not be the only protein modification regulating enolase activity.

A limitation of our work is that our data do not prove a cause-and-effect relationship between decreased glycolytic flux and heart failure in CrT-OE mice. Further experiments in intact hearts could be performed to confirm that glycolytic flux (in addition to capacity) is indeed decreased in this model; however, even these measurements would not establish a causal relationship. One approach to address this might be to cross our CrT-OE mice with GLUT1 over-expression mice [56], which show increased glycolytic capacity, to test whether this prevents the development of LV dysfunction.

In summary, this work shows that CrT-OE mice have chronically elevated levels of creatine over their first year of life. In spite of this, cardiac function is initially normal and declines progressively only after 6 weeks of age. The mechanism for this decline may result from impaired glycolysis at the level of enolase, in part mediated by increased β ERF-1 expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

^1H -MR spectra and creatine contents from wild-type and CrT-OE mice. Representative ^1H -MR spectra from a wild-type (A) and a CrT-OE (B) mouse, obtained from a 2 μl voxel placed in the inter-ventricular septum. The arrow indicates the supra-normal creatine level (CH_3 -resonance of a creatine at 3 ppm). Creatine content was measured using ^1H -MRS at 6 weeks, 16 weeks, 32 weeks, and 52 weeks and by HPLC at 52 weeks (C). * denotes a p-value less than 0.05 for wild-type versus CrT-OE at each time-point. No significant difference was observed for creatine content across the 52 weeks of this study.

Figure 2.

Cine-MR images and function information for wild-type and CrT-OE hearts. Panel A shows *in vivo* short-axis cine-MR images from a wild-type mouse at end diastole (upper left) and end systole (lower left), and from a CrT-OE mouse at end diastole (upper right) and end systole (lower right) at 32 weeks of age. The white bar is equal to 2 mm. Panel B shows the LV ejection fraction for wild-type and CrT-OE mice over the 52 week study period. * denotes a p-value less than 0.05 for wild-type vs CrT-OE (i.e., between groups) and # denotes a p-value less than 0.05 when comparing all parameters over time (i.e., within groups).

Figure 3.

Correlation of myocardial creatine content and cardiac function in CrT-OE mice. Graph A correlates the mean myocardial creatine content over 52 weeks with the ejection fraction at 52 weeks. Graph B correlates the myocardial creatine content at 6 weeks with the decline in ejection fraction from 6 weeks to 52 weeks.

Figure 4.

Proteomics comparison of LV tissue from a wild-type and CrT-OE heart. Panel A shows a representative 2D DIGE image comparing wild-type (labeled red, Cy3) and CrT-OE (labeled green, Cy5) myocardial protein profiles. Proteins are separated in the horizontal direction by isoelectric focusing point from pH ~3 to 10, and vertically by molecular weight from ~150 to 10kD. Panel B shows a magnification of the changes to β-enolase and α-enolase. In addition to a decrease in enolase content in CrT-OE hearts, protein elements of both β-enolase and α-enolase undergo acidic isoelectric shifts, which suggest that a post-translational modification may be acting on these proteins.

Figure 5.

Measurement of enolase activity in wild-type, CrT-OE, sham control, and aortic banded (hypertrophied) hearts. Panel A shows that the rate of ATP produced (which in this assay is directly proportional to enolase activity) is significantly suppressed in CrT-OE hearts relative to wild-type and in aortic banded hearts relative to sham controls, where * and # denote p-values less than 0.01 and 0.05, respectively. Panel B correlates enolase activity with myocardial creatine content.

Figure 6.

Measurement of glycolytic capacity in wild-type and CrT-OE hearts. Panel A demonstrates that the rate of lactate production via anaerobic glycolysis was decreased in CrT-OE heart extracts relative to wild-type controls, where # denotes a p-value less than 0.05. Panel B correlates lactate production with myocardial creatine content.

Figure 7.

Western blot analysis of β-enolase repressor factor 1 for wild-type and CrT-OE hearts. Panel A shows that β-enolase repressor factor 1 is increased in CrT-OE hearts relative to wild-type controls. This example shows a representative Western blot of β-enolase repressor factor 1 (89 kDa) and β actin (43 kDa) in a WT and CrT-OE mouse with high myocardial creatine levels (i.e., above 130 nmol creatine per mg protein). Panel B and Panel C respectively display the β-enolase repressor factor 1 and β actin results quantitatively for 10 WT and 10 CrT-OE hearts, where * denotes a p-value less than 0.01. Panel D correlates β-enolase repressor factor 1 expression with myocardial creatine content.

Table 1
Cardiac Parameters for WT and CrT-OE Mice Measured by MRI

Cardiac parameters for wild-type and CrT-OE mice as measured by cine-MRI.

	6 weeks			16 weeks			32 weeks			52 weeks		
	WT	CrT-OE	WT	CrT-OE	WT	CrT-OE	WT	CrT-OE	WT	CrT-OE	WT	CrT-OE
<i>n</i>	14	26	14	26	14	26	14	26	14	23		
Body wt, g	20±3	20±3	26±5 [#]	26±4 [#]	29±6 [#]	31±6 [#]	31±7 [#]	31±6 [#]	31±7 [#]	34±7 [#]		
LV wt, mg	74±11	76±8	96±16 [#]	100±11 [#]	111±17 [#]	115±13 [#]	118±19 [#]	115±13 [#]	118±19 [#]	151±22 ^{*#}		
LV wt/body wt	3.8±0.3	3.9±0.5	3.8±0.5	3.9±0.6	3.9±0.5	3.9±0.7	3.9±0.5	3.9±0.7	3.9±0.5	4.4±0.7 ^{*#}		
LVEDV, μ L	50±11	51±7	57±16	64±12 [#]	72±15 [#]	73±14 [#]	73±16 [#]	73±14 [#]	73±16 [#]	79±12 [#]		
LVESV, μ L	22±6	24±5	23±10	33±9 ^{*#}	28±10	40±11 ^{*#}	27±10	40±11 ^{*#}	27±10	46±13 ^{*#}		
LVEF, %	56±4	53±5	62±9	49±9 [*]	64±7 [#]	45±9 ^{*#}	64±6 [#]	45±9 ^{*#}	64±6 [#]	43±13 ^{*#}		
Stroke volume, μ L	28±5	27±4	34±8 [#]	31±7 [#]	47±12 [#]	33±8 ^{*#}	48±11 [#]	33±8 ^{*#}	48±11 [#]	32±9 ^{*#}		
Cardiac Output, μ L	14±3	13±2	17±5	16±3 [#]	23±6 [#]	16±4 ^{*#}	23±6 [#]	16±4 ^{*#}	23±6 [#]	16±4 ^{*#}		
Heart rate, bpm	483±27	465±28	480±57	497±51 [#]	483±43	491±44	476±51	491±44	476±51	489±40		

Data are reported as mean $\pm$ STD;

* p<0.05 for WT versus CrT-OE (i.e., between groups) and

p<0.05 compares all parameters over time (i.e., within groups). Abbreviations used are: LV-left ventricle, EDV-end diastolic volume, ESV-end systolic volume, and EF-ejection fraction.

Table 2
Protein Differences between WT and CrT-OE Mice

Myocardial protein differences between wild-type and CrT-OE mice. All values are relative to wild-type animals (i.e., negative means lower protein expression in CrT-OE).

Protein Category	Protein Annotation	Protein ID	2D Gel Fold Change (n=4)	2D Gel p-values	2D Gel Spot Number
Glycolysis	β-enolase	IP100228548.4	-2.81 ± 0.14	0.03	3
	α-enolase	IP100462072.1	-1.71 ± 0.11	0.04	2
Fatty Acid Oxidation	Delta3,5-delta2,4-dienoyl-CoA isomerase, mitochondrial precursor	IP100130804.1	-1.53 ± 0.06	0.02	7
Krebs Cycle	Isocitrate dehydrogenase, mitochondrial precursor	IP100131257.1	1.72 ± 0.12	0.04	4
	Malate dehydrogenase, mitochondrial precursor	IP100323592.1	-1.36 ± 0.06	0.03	5
	Malate dehydrogenase 2, NAD	IP100331590.1	-1.38 ± 0.09	0.04	6
Sarcomeric	β-myosin heavy chain	IP100130653.1	1.81 ± 0.10	0.04	1