Mitochondrial Creatine Kinase Attenuates Pathologic Remodeling in Heart Failure

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Abstract

Background: Abnormalities in cardiac energy metabolism occur in heart failure (HF) and contribute to contractile dysfunction, but their role, if any, in HF-related pathologic remodeling is

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much less established. Creatine kinase (CK), the primary muscle energy reserve reaction which rapidly provides ATP at the myofibrils and regenerates mitochondrial ADP, is down-regulated in experimental and human HF. To test the hypotheses that pathologic remodeling in human HF is related to impaired cardiac CK energy metabolism and that rescuing CK attenuates maladaptive hypertrophy in experimental HF.

Methods: First, in twenty-seven HF patients and fourteen healthy subjects, we measured cardiac energetics and left ventricular (LV) remodeling using noninvasive magnetic resonance ^{31}P spectroscopy and MRI, respectively. Second, we tested the impact of metabolic rescue with cardiac-specific overexpression of either myofibrillar CK (CKmyofib) or mitochondrial CK (CKmito) on HF-related maladaptive hypertrophy in mice.

Results: In people, pathologic LV hypertrophy and dilatation correlate closely with reduced myocardial ATP levels and rates of ATP synthesis through CK. In mice, transverse aortic constriction (TAC)-induced LV hypertrophy and dilatation are attenuated by overexpression of CKmito, but not by overexpression of CKmyofib. CKmito overexpression also attenuates hypertrophy after chronic isoproterenol stimulation. CKmito lowers mitochondrial ROS, tissue ROS levels, and upregulates antioxidants and their promoters. When the CK capacity of CKmito-overexpressing mice is limited by creatine substrate depletion, the protection against pathologic remodeling is lost, suggesting the ADP regenerating capacity of the CKmito reaction rather than CK protein *per se* is critical in limiting adverse HF remodeling.

Conclusions: In the failing human heart, pathologic hypertrophy and adverse remodeling are closely related to deficits in ATP levels and in the CK energy reserve reaction. CKmito, sitting at the intersection of cardiac energetics and redox balance, plays a crucial role in attenuating pathologic remodeling in HF.

Clinical Trial Registration: NCT00181259

INTRODUCTION:

Heart failure (HF) remains a significant, growing worldwide public health problem. More Medicare dollars are spent on the care of HF than on any other diagnosis.[1] Adverse outcomes in HF are associated with two related but separate clinical features: contractile dysfunction and adverse remodeling, the latter characterized by the pathologic ventricular hypertrophy and dilatation. Contractile dysfunction in experimental and human HF has been extensively studied and is related, at least in part, to inadequate ATP energy metabolism.[2-4] However, the role of energetic abnormalities, if any, in contributing to adverse remodeling is far less understood. On the one hand, reductions in energy-related metabolites in the failing heart may be a *consequence* of adverse remodeling since ventricular dilatation increases wall tension, a determinant of energetic demand. On the other hand, energetic abnormalities precede adverse remodeling in experimental HF, suggesting but not proving, that impaired energetics may *cause* adverse remodeling.[5,6]

The energy liberated during ATP hydrolysis fuels nearly all myocellular functions. Most myocardial ATP is generated primarily via mitochondrial oxidative phosphorylation.[6] Yet the creatine kinase (CK) reaction is the primary short-term high-energy phosphate energy supply[2,7] in that it rapidly and reversibly generates ATP by phosphoryl transfer from creatine phosphate (PCr) to adenosine diphosphate (ADP) in the cytosol and the reverse reaction in the mitochondria.[7,8] Abnormalities in CK activity and metabolites occur in experimental and human HF[9-13], and reductions in cardiac function and work are closely related to impaired CK ATP transfer in human HF.[14] Surprisingly, little is known about the role that impaired CK energy metabolism plays in adverse cardiac remodeling. Transgenic over-expression of the cytosolic, myofibrillar CK (CKmyofib), rescues depressed cardiac CK energy metabolism, improves contractile function, and prolongs survival in several murine models of HF.[15,16] Yet, CKmyofib overexpression does not attenuate the development of left ventricular hypertrophy (LVH) or pathologic dilatation.[15] The mitochondrial isoform of CK (CKmito) is less abundant and has been far less investigated in HF, despite its strategic location in the mitochondrial intermembrane space where it plays a critical role in energy transfer and stimulating oxidative phosphorylation by regenerating mitochondrial ADP.[17,18] In theory, CKmito could enhance ATP production and limit the formation of reactive oxygen species (ROS), which can contribute to pathologic hypertrophy and remodeling.[19]

We set out to determine whether abnormalities in cardiac energetics and specifically in mitochondrial CK, contribute to adverse HF remodeling. We find that both LVH and dilatation in human HF are closely associated with reduced *in vivo* myocardial ATP concentrations, [ATP], and the rate of ATP synthesis through CK. In a mouse model of HF induced by transverse aortic constriction (TAC), cardiac-specific overexpression of CKmito prevents TAC-induced decline in *in vivo* cardiac energetics, modestly enhances contractile function, and significantly limits the development of LVH and ventricular dilatation. In contrast, overexpression of the cytosolic, myofibrillar CK isoform did not limit LVH or ventricular dilatation. Mitochondrial CK overexpression reduces mitochondrial ROS, attenuates the increase in left ventricular (LV) ROS following TAC, upregulates several antioxidant enzymes in failing murine hearts, and increases myocyte tolerance to exogenous oxidative stress. The expression of several pro-hypertrophic signaling molecules is attenuated with CKmito overexpression while that of the regulator, protooncogene, cMyc, is suppressed by CKmito but not by CKmyofib overexpression. When ATP flux through the CK reaction is reduced by depleting creatine, a CK substrate, CKmito overexpressing mice are no longer protected from pathologic hypertrophy and adverse remodeling following TAC. The results demonstrate that the mitochondrial ADP regenerating role of CKmito and not the protein, per se, is vital for attenuating pathologic cardiac remodeling. Taken together, the data indicate that CKmito resides at the interface of subcellular energetics, redox balance, and adverse remodeling in the failing heart. Thus, strategies that maintain mitochondrial CK activity may offer a metabolic approach for reducing pathologic HF remodeling, its consequences, and attendant morbidity and mortality.

METHODS

Data availability:

The data that support the findings of this study are available from the corresponding author upon reasonable request. The authors have full access to all the data in the study and take responsibility for its integrity and the data analysis.

Study subjects:

The study was performed at the Johns Hopkins Hospital, approved by its Institutional Review Board, and all participants provided written consent. Twenty-seven patients (age = 45±14 yrs, mean±SD) with a history of HF (New York Heart Association, NYHA, class I-III) and reduced LV EF (45%) were enrolled, as previously described.[14] Fourteen age-matched healthy subjects (age = 42±18 yrs) with no heart disease history, diabetes, or hypertension were enrolled as controls. The initial report of these subjects focused on cardiac work and contractile abnormalities and contained additional demographic information.[14] After publication, the data were subsequently analyzed to test for a relationship between altered energy metabolism and adverse remodeling. That previously unpublished analysis appears here. Further details appear in the Supplement.

Transgenic mouse lines:

Two types of mice were generated: first, those with a transgene for the regulatory protein tTA (tetracycline-controlled transactivator) under the control of the α-MHC promoter; and second, mice expressing the CKmito transgene under the control of tetracycline-responsive element (TRE) following microinjection of the CKmito construct into fertilized mouse embryos (C57BL/6 strain). After crossing of TRE-CKmito mice with α-MHCtTA mice, double transgenic mice (CKmito-tTA) were confirmed by genotyping, and CKmito transgene induction was achieved by a diet free of doxycycline (designated CKmito overexpressors). Control mice (hereafter called "WT") were littermates containing either the tTA or CKmito transgene alone. Guanidinoaceate N-methyltransferase knock-out (GAMT$^{-/-}$) mice were created as previously described and provided as a gift.[20] Animals were assigned an alphanumeric code so that investigators were blinded to the genetic background and group assignment of each mouse. Additional details appear in the Supplement.

Interventions to induce in vivo hypertrophy/heart failure:

Eight to 16-week-old male mice underwent sham or TAC surgery as previously described.[5,15] Chronic isoproterenol administration was accomplished by subcutaneous implantation of Alzet osmotic mini-pumps containing either 0.0125mg/kg of isoproterenol or saline. Details appear in the Supplement.

Animal experiments:

The methods for all experiments are described in detail in the Supplement. A statistical power analysis identified the target number of animals to demonstrate significance at an

expected difference of roughly 25% and a 15% variation (based in part on CK flux and LV mass differences between groups).

Statistical Analysis:

All statistical analyses are summarized in the Online Supplement and specific tests and results show in Supplement Table SI.

RESULTS

Left ventricular hypertrophy and adverse remodeling in human heart failure are associated with reduced myocardial ATP levels and rates of ATP synthesis through cardiac CK

Contractile dysfunction in human HF is associated with impaired myocardial energy metabolism[14]. However, a link between adverse remodeling, another well-recognized consequence of myocardial injury, and impaired myocardial energy metabolism has not been studied in the failing human heart. First, we measured cardiac energetics and LV remodeling in twenty-seven patients with HF using noninvasive phosphorus ^{31}P MRS and ^{1}H cine MRI, respectively, and compared the results with those of fourteen healthy subjects. HF patients had a clinical history of HF, contractile dysfunction with LV ejection fraction (EF) 45%, and no history or findings of significant ischemic heart disease or infarction, factors known to disrupt mitochondrial function and potentially exaggerate any perceived role of impaired energy metabolism. As previously reported in these subjects,[14] myocardial mass and end-diastolic volumes (EDV) were increased, and [ATP] and the rate of ATP synthesis through CK were reduced in HF patients as compared to healthy subjects (Fig 1A-D). The novel and previously unpublished observations here are that critical indices of pathologic hypertrophy (measured by LV mass) and of ventricular dilatation (measured by EDV) correlated significantly with lower *in vivo* myocardial [ATP] and rates of ATP synthesis through myocardial CK (Fig 1E-H). This new evidence that established indices of adverse remodeling in human HF are closely correlated with reductions in simultaneous *in vivo* measures of ATP and myocardial CK energetics suggests a role for reduced CK energy metabolism in adverse remodeling in human HF.

Mitochondrial CK counters maladaptive cardiac hypertrophy

These human data do not distinguish the contributions of different CK isoforms to *in vivo* CK activity (as is the case for all prior in vivo ^{31}P MRS studies) and they do not demonstrate whether impaired cardiac energetics (lower ATP and/or CK flux) contribute to pathologic hypertrophy and adverse remodeling or is simply one of many consequences. To probe this relationship, we studied a murine model of HF induced by TAC that results in progressive LVH, contractile dysfunction, and eventual dilatation, as well as energetic and CK abnormalities similar to those observed in human HF.[12,13] The goal was to determine whether attenuating the decline of mitochondrial CK in the failing heart by transgenic CKmito overexpression improves energetics and limits adverse remodeling.

Mice conditionally overexpressing cardiac-specific mitochondrial CK were created and exhibited CKmito protein levels nearly three-fold in wild-type (WT) mice (Fig 2A). The additional CKmito was confirmed to be co-localized to the mitochondria, and the levels

of CKmyofib and CKB isoenzymes were unchanged (Supplement S1A-C). CKmito hearts at baseline exhibited no changes in myocardial mass, dimensions, or energetics, apart from increased *in vivo* rates of ATP synthesis through CK (Fig 2). However, 9-10 weeks following TAC surgery, CKmito hearts exhibited less energetic decline *in vivo* and higher ATP synthesis rates through CK than did WT TAC hearts (Fig 2F-I). This improved energetic status in CKmito TAC hearts occurred with modestly better *in vivo* contractile function and significantly less LVH and dilatation than in WT TAC (Fig 2J-M). Specifically, mean LV mass and EDV following TAC were about 30% and 50% lower, respectively, in CKmito than in WT hearts (Fig 2JK). In contrast, the overexpression of the myofibrillar CK isoform did not attenuate the increase in LV mass or adverse remodeling (EDV) after TAC as previously reported, but the data are shown here for the first time (Fig 2NO).[15]

At 9-10 weeks post-TAC (Fig 2), WT TAC mice exhibit cardiac dysfunction (EF), LV remodeling, and reductions in cardiac CK flux similar to that present in ambulatory patients with NYHA Class II-III heart failure (Fig 1). To gain insight into the role of CKmito overexpression at an earlier stage of pathologic remodeling, we also studied mice just 2-3 weeks following TAC. In these studies, we observed less hypertrophy (LV mass/body weight) and remodeling (LVID/body weight) and a trend for higher EF (p=0.080) in CKmito TAC versus control TAC (Supplement S1D-H). Thus, there is a signal for CKmito overexpression protection even at the earliest stages of pathologic remodeling in 2-3 week TAC hearts (Supplement S1D-H) and significant attenuation of pathological remodeling and improved function at 9-10 weeks post-TAC (Fig 2) when control animals exhibit remodeling, function, and metabolism similar to the findings of patients with heart failure.

To determine whether CKmito overexpression also attenuates hypertrophy in other pathologic settings, we observed that the increase in LV mass resulting from the chronic infusion of the β1/β2 agonist, isoproterenol (ISO), was also significantly attenuated by CKmito overexpression (Fig 2P). Because ISO did not cause LV dilatation in WT over the time interval studied (Fig 2Q), the remainder of the experiments focused on the 9-10 week TAC model that manifests both pathologic hypertrophy and ventricular dilatation (remodeling) akin to that observed in human HF. Nevertheless, the ISO experiments are important because ISO is an established model of LVH and the findings demonstrate that attenuation of LVH by CKmito preservation is not limited to a single model. Thus, attenuating the energetic decline in failing hearts by overexpressing CKmito improves *in vivo* ATP synthesis through cardiac CK, high-energy phosphate levels, and LV function and significantly mitigates LV hypertrophy development in two models and limits pathologic dilatation after TAC. Attenuation of hypertrophy and dilatation after TAC was not observed with myofibrillar CK overexpression. Taken together, we interpret these findings to indicate a specific role for CKmito in limiting pathologic hypertrophy and adverse remodeling.

Mitochondrial CK limits myocardial redox imbalance in HF

Oxidative stress can be a significant determinant of pathologic remodeling, and the mitochondria are a substantial source of endogenous ROS. To determine whether TAC exacerbates ROS production in the heart, and if so, whether CKmito overexpression counters this effect, we assessed myocardial tissue oxygen-derived free radicals using

electron paramagnetic resonance (EPR) (Fig 3A). We observed a significant increase in ROS following TAC in WT LV but not in mice overexpressing CKmito (Fig 3AB). To determine whether overexpressing CKmito renders the cardiac myocyte more resistant to ROS, we exposed myocytes isolated from WT and CKmito mice to exogenous H_2O_2. We found that survival after H_2O_2 exposure was shortened by TAC in WT myocytes. However, survival after H_2O_2 in TAC CKmito myocytes was not shortened and was not significantly different than that of sham myocytes (Fig 3C). Next, we measured major cellular antioxidants, starting from the thioredoxin (Trx) system, since thioredoxin 1 is also a well-known endogenous negative regulator of cardiac hypertrophy.[21] Because the efficiency of Trx in quenching H_2O_2 via peroxiredoxins is determined by the activity of thioredoxin reductase (TrxR),[22] we measured the levels of TrxR protein abundance and total TrxR activity. We found both TrxR2 expression and total TrxR activity significantly enhanced after TAC in the CKmito hearts (Fig 3D-F). To better understand why TrxR protein and activity are increased in CKmito hearts, we assessed nuclear factor erythroid-2 related factor 2 (Nrf2), a master regulator of the endogenous antioxidant defense system that transcriptionally regulates the cellular Trx system (i.e. Trx and TrxR, in particular TrxR1).[23] We observe that Nrf2 is significantly upregulated even at baseline in CKmito hearts (Fig 3G). Superoxide dismutase (SOD) content was higher in CKmito hearts before TAC (Fig 3H). This set of data shows that resistance to ROS is lower in TAC-failing myocytes but maintained in those from CKmito overexpressing hearts and that the latter occurs with, and likely results from, an increase in antioxidant proteins. In concert, there is a significant increase in tissue ROS levels with TAC in WT failing hearts but not in CKmito overexpressors.

CKmito overexpression improves mitochondrial function and reduces proton leak

Mitochondria are a primary source of intracellular ROS and host the mitochondrial CK isoform. The *in vivo* data above demonstrate improved energy balance in CKmito overexpressing failing hearts (Fig.2), suggesting a central role for mitochondria. Therefore, we investigated isolated mitochondrial function, also to rule out possible confounding extra-mitochondrial factors, such as energy demand differences under hemodynamic load. We observed that total mitochondrial mass is likely unchanged in CKmito overexpressing mice, as evidenced by unchanged citrate synthase activity (Fig 4A). We assessed mitochondrial respiration supported by substrates of Complex I (Glutamate/Malate), Complex II (Succinate/Rotenone) and Complex IV (TMPD). TAC reduced ADP-stimulated (State 3) respiration in WT mitochondria for both Complexes I and II. However, there was no statistically significant decline in Complex I or Complex II after TAC in CKmito overexpressing mitochondria. There was no statistically significant difference in Complex IV activity among the groups, ruling out any artifacts related to aberrant loading or mitochondrial damage during isolation. In addition, mitochondrial proton leak (in the presence of Oligomycin to block the ATP Synthase) was significantly lower, at baseline and with TAC, in mitochondria from CKmito overexpressors (Fig 4I) while maximal uncoupled respiration also did not differ statistically across groups (Fig 4J). Thus, CKmito overexpression prevents TAC-induced mitochondrial function decline and improves coupled respiration.

Next, we directly measured ROS in isolated mitochondria. Mitochondrial ROS emission was significantly lower at baseline in CKmito overexpressors than in WT mitochondria and declined with TAC in both CKmito and WT (Fig 4K). Of note, when the TrxR inhibitor, auranofin, was administered, mitochondrial ROS emission increased dramatically in CKmito OE but not WT mitochondria, indicating a critical role for augmented TrxR in CKmito overexpressing mitochondria in buffering mitochondrial ROS (Fig 4L).

To assess the functional implications of these biochemical findings, we challenged myocytes obtained from WT and CKmito mice, at baseline or after TAC, with isoproterenol (ISO). We found that ISO-induced contractile response was severely blunted in isolated WT TAC myocytes but not in the CKmito overexpressing TAC cells (Fig 4B). Since ROS can oxidatively modify proteins implicated in myocyte Ca^{2+} handling and thereby reduce Ca^{2+} cycling, we also measured myocyte whole-cell Ca^{2+} transients after ISO stimulation. The Ca^{2+} transient response to ISO declined in WT TAC myocytes (Fig 4C) but was preserved in CKmito TAC myocytes, compared to their respective non-failing controls. In summary, the overexpression of mitochondrial CK does not result in a statistically significant change in mitochondrial mass but preserves Complex I and II-supported oxidative phosphorylation after TAC and reduces proton leak, offsetting the energetic deficits observed in HF.[24,25] These favorable events likely account for the preserved Ca^{2+}-cycling and contractile reserve seen in TAC CKmito myocytes upon ß-adrenergic challenge. In addition, CKmito overexpression significantly reduces mitochondrial ROS at baseline, through enhanced TrxR mechanisms.

Local energy recycling mechanisms are required to explain CKmito anti-hypertrophic effects: evidence from creatine deficient mice

First, to test the hypothesis that limiting the ADP regenerating capacity of CKmito worsens pathologic remodeling, we used mice deficient in creatine, a substrate for the CK reaction, and compared creatine-deficient guanidinoacetate N-methyltransferase knock-out (GAMT$^{-/-}$) mice with control mice following TAC. Creatine-deficient GAMT$^{-/-}$ mice were smaller than WT mice and exhibited worse LV function following TAC (EF, p=0.060) and more pathological remodeling with higher LV mass (p=0.0028) and EDV (p=0.072) than WT TAC mice when corrected for body weight (Supplement S2D-E). LV ROS levels were also higher in creatine-deficient GAMT$^{-/-}$ mice than in WT both at baseline and following TAC (Supplement S2G).

Second, to distinguish whether the impact of CKmito overexpression to limit pathological LV remodeling is tied to its well-known energetic effects of regenerating ADP for mitochondrial ATP synthesis or due to other "non-metabolic" stabilizing effects of the CKmito protein *per se*, we pursued orthogonal experiments. We speculated that, given its compartmentalization, CKmito might have protein membrane-stabilizing and other effects that contribute to the attenuation of pathologic hypertrophy and adverse remodeling, independent of its ability to regenerate ADP.[26] To answer this question, we crossed CKmito-overexpressing mice with creatine-deficient guanidinoacetate N-methyltransferase knock-out (GAMT$^{-/-}$) mice. The resultant double transgenic mice (GAMT$^{-/-}$xCKmito) exhibited increased CKmito protein content but lower ATP flux through the CK reaction

due to CK substrate depletion. ATP synthesis through cardiac CK in double transgenics was not detectable by *in vivo* ^{31}P MRS studies, as anticipated. Unlike the observations in CKmito-overexpressing mice (Fig 2JK), CKmito overexpression in GMAT$^{-/-}$ mice did not attenuate the development of pathologic hypertrophy or adverse remodeling when the CK reaction was inhibited by creatine substrate depletion (Fig 5AB) in that *in vivo* LV mass and EDV did not differ between GAMT$^{-/-}$xWT *vs.* GAMT$^{-/-}$xCKmito hearts following TAC (Fig 5BC). Likewise, the TAC-induced increase in tissue ROS was no longer attenuated, and intrinsic antioxidants were no longer augmented when the CK energy reaction was substrate-limited in CKmito-overexpressors (GAMT$^{-/-}$xCKmito *vs.* GAMT$^{-/-}$xWT hearts, Fig 5). Thus, overexpressing the CKmito protein was ineffective in countering ROS bursts and preventing hypertrophy and adverse remodeling when ADP regeneration and ATP recycling through mitochondrial CK were attenuated by creatine substrate deprivation. Local energy production and recycling by CKmito in the failing heart, and not just the presence of the protein, is required for attenuating pathologic hypertrophy and adverse remodeling.

CKmito enhances the balance of pro-/anti-hypertrophic transcription factor in pressure overloaded hearts

The links between cardiac energetics, redox balance and transcription factors previously reported to regulate myocardial hypertrophy are likely complex and incompletely understood. Thus, as both a hypothesis-generating endeavor and to place in context how the new CKmito energetic observations above relate to the considerable prior literature on molecular signaling in the development of hypertrophy, we evaluated changes in several hypertrophic signaling candidates. Besides the Trx system reported above, at least two other systems are known to sit at the intersection between hypertrophy and cardiac redox conditions. These are forkhead box O3 (FOXO3) and the protooncogene, cMyc. We observed that several hypertrophic transcription factors tracked in similar directions in both CKmito and CKmyofib overexpressing hearts despite the differing impact of CKmito and CKmyofib on pathologic hypertrophy, including protective FOXO3a and Mad1 which were both higher in CKmito and CKmyofib overexpressors than in WT following TAC (Supplement S3). Likewise, we examined the status of another ROS-sensitive transcription factor, GATA-4, and found that, in agreement with previous reports,[27,28] GATA-4 expression is markedly upregulated in WT TAC hearts but not in CKmito or CKmyofib mice (Supplement S3). In contrast, we observed that the expression of cMyc is increased in WT hearts after TAC, consistent with previous findings,[29-34] but that this up-regulation is attenuated in the presence of CKmito overexpression (Fig 6A) and not attenuated by CKmyofib overexpression (Fig 6B).

Finally, we tested whether, in addition to the direct modulation of pro- and anti-hypertrophic redox-sensitive genes, CKmito could also interfere with post-translational modifications of kinases involved in the regulation of myocardial growth in response to stress. Phosphorylated and total mammalian target of rapamycin (mTOR) and mitogen-activated protein kinase (ERK), did not differ between WT and CKmito hearts (Supplement S4). Thus, CKmito overexpression attenuates the TAC-induced increase in redox-dependent activation of pro-hypertrophic signaling (c-Myc and GATA-4), boosts key antioxidant

defenses with anti-hypertrophic properties (Trx), and attenuates the decline in protective FOXO3 levels, factors at the crossroads of oxidant stress and nutrient signaling.

DISCUSSION

This work demonstrates for the first time to our knowledge that pathologic hypertrophy and adverse remodeling in human HF directly correlate with decreased *in vivo* myocardial ATP levels and reduced rates of ATP synthesis through CK, the primary muscle energy reserve reaction. While such energetic changes could, in theory, be a consequence of hypertrophy and adverse remodeling, more basic murine studies here demonstrate that reductions in the mitochondrial isoform, but not the myofibrillar isoform, of CK, contribute to pathologic hypertrophy and remodeling. The mitochondrial CK isoform was previously reported to be reduced in experimental HF across species and in human HF.[8,35,36] We now show that augmenting mitochondrial CK limits murine pathologic hypertrophy resulting from either chronic adrenergic stimulation or that due to TAC. The murine TAC HF model manifests similar contractile and energetic characteristics to those of human HF,[5,12,15] and transgenic overexpression of cardiac mitochondrial CK limits the declines in cardiac ATP and the rate of ATP synthesis through CK. Importantly, it mitigates the development of pathologic hypertrophy and adverse ventricular dilatation. This is in contrast to attenuating the loss of the cytosolic myofibrillar form of CK (CKmyofib) which, in a prior report, did not alter pathologic remodeling.[15] These new data also signal that CKmito overexpression attenuates earlier stages of pathologic hypertrophy, such as those observed after only 2-3 weeks after TAC (Supplement S1D-H).

To better understand the mechanism by which the mitochondrial CK isoform limits pathologic hypertrophy and remodeling, we first measured tissue ROS, a potent stimulus of pathologic hypertrophy often produced in mitochondria and increased by a dysfunctional mitochondrial mismatch of oxidative metabolism and ATP synthesis. We observed that CKmito overexpression attenuates the increase in left ventricular tissue ROS during HF and affords isolated myocytes increased resistance to exogenous ROS-induced cell death. Our data also show that CKmito augments antioxidant defenses, as demonstrated by augmented TrxR protein and activity and increased SOD, but also lowers mitochondrial ROS emission and improves *in vivo* and *in vitro* mitochondrial function in the failing heart. The importance of metabolic function is demonstrated by the observation that limiting CKmito ADP regeneration by depleting creatine, worsens pathologic remodeling (Supplement S2) and negates the ability of CKmito overexpression *per se* to attenuate pathologic hypertrophy in the failing heart (Fig 5A-B). The intersection of cardiac CK and several established pro-hypertrophic signaling pathways were studied. Although several transcription factors were unchanged by CKmito overexpression, the increase in pro-hypertrophic and ROS sensitive c-Myc was attenuated by CKmito overexpression, but not by CKmyofib overexpression in failing hearts. We interpret all of these data taken together to indicate that pathologic hypertrophy and adverse remodeling, established predictors of adverse clinical HF outcomes and mortality, are closely associated with reduced ATP levels and synthesis through CK in the failing human heart. Moreover, CKmito, which sits at the intersection of cardiac mitochondrial ADP regeneration and pathologic ROS generation, plays a critical role in

maintaining energy metabolism, limiting ROS, and mitigating pathologic hypertrophy and adverse remodeling in the failing heart.

Multiple myocardial energetic metabolic pathways are altered in failing hearts, and several offer potential metabolic treatment targets.[4,37] The hydrolysis of ATP is the biochemical energy utilized to sustain normal myocardial contractile and relaxation function and to fuel most myocellular processes. Cardiac ATP synthesis rates are the highest per gram of any organ in the body[2], but because cardiac ATP stores are small relative to the demand, increases in energy demand due to augmented myocardial work or hypertrophy must be tightly matched by increased ATP synthesis.[38] Most ATP is generated by phosphorylation of ADP, primarily via mitochondrial oxidative phosphorylation.[39] The CK reaction is a more rapid source of ATP than that provided by oxidative phosphorylation. It is often considered the primary myocardial high-energy phosphate reserve reaction called upon during exercise and other forms of stress.[2] CK rapidly and reversibly generates ATP at the myofibrils by phosphoryl transfer from creatine phosphate (PCr) to ADP and the reverse reaction generates ADP in the mitochondria with as much as 90% of ATP moving through CK.

Prior lines of experimental and human evidence link reduced ATP generation by the CK reaction to systolic and diastolic dysfunction, but the role of CK, if any, in adverse remodeling in HF was far less established. The former include observations that inhibiting or reducing CK in healthy hearts adversely limits contractile reserve,[40,41] that reductions in CK metabolites and/or ATP synthesis through CK (hereafter termed "CK flux") impair diastolic function in healthy, non-failing hearts,[42,43] and that reduced CK metabolism (i.e., decreased CK flux, [ATP], [PCr], [Cr] and total CK activity) is observed in nearly all experimental HF models and in human HF.[44-46] Most of our understanding of human myocardial high energy phosphate metabolism comes from studies using ^{31}P MRS, the only noninvasive means to quantify high energy phosphates in the beating heart. *In vivo* ^{31}P MRS saturation transfer studies identified more severe decreases in CK flux than in PCr/ATP in HF patients.[11-13,47] However, those prior reports did not study remodeling[12] or studied it in a different patient population (patients with hypertrophic cardiomyopathy of which only five had reduced ejection fraction) and did not find a significant relationship of CK flux with remodeling.[13] Reductions in cardiac CK flux in the failing human heart are now recognized as independent predictors of adverse clinical HF outcomes[48] and were more recently shown to have a close association with reduced contractile work and performance.[14] In terms of the potential link between reduced CK energetics and adverse remodeling in HF, adverse remodeling following coronary ligation in pigs in the absence of HF was associated with reduced PCr/ATP whereas pigs with both adverse remodeling and HF had the lowest cardiac PCr/ATP.[45] Moreover, in a porcine model of pressure-overload, the degree of hypertrophy was inversely related to cardiac PCr/ATP and the amount of both the myofibrillar and mitochondrial isoforms of CK, while the mitochondrial isoform content correlated directly with the rate of ATP synthesis through CK.[49] Finally, reductions in cardiac PCr/ATP in the murine TAC model predict subsequent pathologic remodeling as evidenced by serial changes in EDV.[5] In addition, it should be noted that myocardial PCr and ATP concentrations were directly measured with ^{31}P MRS in both the human and murine studies in the current manuscript. Contemporary human ^{31}P MRS studies that assume ATP concentrations or rely

solely on PCr/ATP could underestimate the extent of metabolic abnormalities and potentially miss ATP depletion, here closely linked to pathologic remodeling.

Reductions in each CK isozyme with HF may have different consequences, although they catalyze the same reaction and have extensive sequence homology. The predominant CK isozymes in the healthy adult heart, CKmyofib and CKmito, are expressed in a tissue-specific fashion, have distinct intracellular locations, and differ in their activity.[50,51] CKmyofib is a dimer that occurs in the cytosol, binds tightly to the M-band, and as such, is thought to represent a functional micro-compartment that facilitates myofibrillar ATP transfer and under physiologic conditions produces ATP.[51] CKmito occurs predominantly as an octamer found in the mitochondrial intermembrane space in close proximity with the adenine nucleotide translocator and the mitochondrial periphery with the mitochondrial permeability transition pore complex (Fig 6C).[50] CKmito under physiologic conditions results in ADP regeneration and is particularly sensitive to inactivation by oxidative injury[50,52-54], which is associated with apoptosis.[50] In HF models and human HF, the amount of CKmyofib and CKmito both decrease from normal, non-failing levels[8,55]. As noted above, the reduction in CKmito is associated with reductions in CK flux in experimental HF.[49] Overexpression of cytosolic CKmyofib in the same TAC HF model used in the present studies improved *in vivo* cardiac energetics, LV systolic function, and survival[15] but did not attenuate adverse remodeling (Fig 2NO). Conversely, overexpression of CKmito, shown here for the first time, attenuated adverse remodeling by limiting the increase in LV mass (Fig 2J) and ultimately attenuating an increase in EDV (Fig 2K) in hearts 9-10 week post TAC. CKmito overexpression also limited early pathologic remodeling after only 2-3 weeks following TAC (Supplement S1D-IH) and significantly attenuated the increase in LV mass following chronic adrenergic stimulation as well (Fig 2P). The ability of CKmito overexpression to differentially attenuate pathologic hypertrophy and adverse remodeling, as compared to CKmyofib overexpressors, is not due to an enhanced ability to increase in vivo CK flux to higher levels at baseline (4.49 ± 1.20 vs 4.15 ± 0.57 umol/g/sec, CKmyofib vs CKmito respectively, p=.48) or following TAC (2.70 ± 0.86 vs 2.31 ± 0.46, umol/g/sec, p=.32, CKmyofib vs CKmito respectively, Fig 2 and ref. 15).

The mechanism by which CKmito attenuates pathologic hypertrophy and adverse remodeling is related, at least in part, to its ability to regenerate ADP in the mitochondria. Depletion of the CK substrate, creatine, worsened pathologic remodeling in GAMT$^{-/-}$ TAC vs WT TAC mice (Supplement Figure S2A-E). Further, inhibition of the ADP regenerating capacity of the CK system by creatine substrate depletion completely abrogated the ability of CKmito overexpression to limit hypertrophy and ventricular dilatation *in vivo* (Fig 5AB, GAMT$^{-/-}$). Thus, there was no evidence that "non-metabolic" or mitochondrial stabilizing effects of CKmito protein, per se, contributed to the attenuation of pathologic hypertrophy and adverse remodeling, despite its location spanning the inner and outer mitochondrial membranes. There is prior evidence that at least in the brain, CKmito plays a vital role as an antioxidant to reduce oxidative stress, and this ability is related to its capacity for ADP regeneration.[26] Moreover, ROS and oxidative stress are well-established contributors to pathologic cardiac hypertrophy.[56] We report here for the first time that CKmito overexpression attenuates the increase in tissue ROS that occurs in WT hearts

following TAC and, importantly, improves myocyte survival following exogenous ROS exposure (Fig 3). We also observe that CKmito impacts myocardial redox balance in at least three ways. First, boosting CKmito helps maintain better mitochondrial coupling and less proton leak in the failing heart, as evidenced by the preserved Complex I/enhanced Complex II activities and reduced proton leak (Figure 4). The additional CKmito enhances ADP availability for ATP synthesis and along with improved Complex I/II and reduced proton leak improves coupling efficiency between respiratory oxygen to ADP phosphorylation and ATP generation, while reducing ROS generation (Fig 6C, scheme). Second, we show that CKmito overexpression reduces mitochondrial ROS (Fig 4K). Third, several major antioxidant pathways are increased with CKmito overexpression at baseline or in response to TAC, including Trx and SOD. In addition, several ROS-related pro-hypertrophic and anti-hypertrophic signaling molecules are down- or upregulated in CKmito hearts, respectively, as discussed below.

The link between transcription factors regulating myocardial hypertrophy, cardiac energetics and redox (i.e., ROS/antioxidant balance) conditions is complex and has yet to be fully elucidated. Besides the Trx system, at least two other systems are at the intersection between hypertrophy and cardiac redox conditions, and both of these involve cardiac metabolism as well: forkhead box O3 (FOXO3) and the protooncogene, cMyc. FOXO3a limits myocardial hypertrophy[57], whereas cMyc promotes it.[31,32] Of note, an inter-relationship exists between cMyc and ROS/tissue redox conditions, while FOXO3a is a well-known suppressor of myocardial hypertrophy[58] and cMyc activity.[59] FOXO3a directly antagonizes cMyc at promoters of nuclear-encoded mitochondrial genes,[59] and via the suppression of cMyc-dependent genes, such as Mxi1 which belongs to the Mad/Mxd family.[60] Moreover, FOXO3a sits at the intersection of oxidant stress[61] and insulin/growth, and enhances antioxidant signaling.[61] Here, we find that CKmito overexpression limits the TAC-induced rise in GATA-4 and cMyc. At the same time, CKmito nearly abrogates TAC-induced decline in FOXO3a. Taken together, the present data indicate that preserving CKmito in a heart prone to failure blunts ROS levels, increases tolerance to exogenous ROS, and likely does so by ameliorating mitochondrial dysfunction at different sites, enhancing mitochondrial ADP regeneration and reducing mitochondrial ROS. It should be emphasized that CKmito and CKmyofib overexpressing hearts exhibit no significant differences in ROS attenuation (Fig 3B and Supplement S5) and increased ROS tolerance (Fig 3C and Supplement S5) as well as FOXO3 and GATA4 changes (Supplement S3). Still, only CKmito attenuates increases in cMyc following TAC (Fig 6). This suggests that cMyc, the protooncogene sitting at the intersection of metabolism, redox, and hypertrophy, may contribute to mitochondrial CK attenuation of pathologic hypertrophy and remodeling. This latter evidence calls for more in-depth studies to define how CKmito differentially regulates various transcription factors.

CKmito overexpression in murine TAC hearts was recently reported to improve *in vivo* cardiac energetics (PCr/ATP) and show a trend of increased survival (p=0.08) but did not attenuate hypertrophy as measured by two-dimensional echocardiography six-weeks after TAC.[62] There are three main differences between that study and the present work that may explain why we identified attenuated adverse remodeling with CKmito overexpression. First, the degree of hypertrophy and extent of adverse remodeling were higher in the current study, which may have afforded a higher likelihood of detecting differences. The

echocardiography measures in the prior investigation were obtained only six weeks after TAC when the LVH had increased by ~60% and the ventricular dilatation by only ~25% in WT animals. However, in the current study, these were several-fold higher at 9-10 weeks post-TAC in WT animals, with an increase in LV mass by ~120% and ventricular dilatation by ~100% (Fig 2). Second, anatomic LV measures by MRI may be more sensitive and reproducible in HF than those by echocardiography since the former are volumetric and don't rely on geometric assumptions. Thus the combination of more significant hypertrophy and remodeling measured with a volumetric imaging modality in the present study may have improved the detection of attenuated pathologic hypertrophy and remodeling in CKmito overexpressing hearts. Even so, we were able to detect a signal for attenuation of hypertrophy with CKmito overexpression at only 2-3 weeks after TAC by echocardiography (Supplement S1HI). Third, the amount of CKmito protein overexpression was higher in the current study (~3x WT) compared to the prior report (~1.5x WT).[62,63] Thus robust, not marginal, CKmito-expression (~3x WT), as shown here, attenuates pathologic hypertrophy and adverse remodeling in the failing heart.

The present findings are novel, but there are limitations to acknowledge. The human studies were performed in a relatively small sample size (41 subjects). However, the cohort was still sufficient to detect significant reductions in cardiac [ATP] and CK flux between HF and healthy subjects and to identify their relationship with increased LV mass and dilatation. Because there are no demonstrated metabolic interventions which specifically and safely manipulate only a single CK isoform in the human heart, we performed studies in mice where specific CK isoform overexpression can be accomplished. Even though overexpression of CKmito improved cardiac energetics in TAC hearts, it did not fully restore ATP levels or rates of flux through CK to those of non-failing hearts. Thus the full impact of CKmito on pathologic hypertrophy and adverse remodeling may be underestimated by these studies. Alternative strategies to better limit the decline and oxidative damage to CKmito should be investigated. Although we characterized the ADP regenerating and ATP exchange properties of CKmito in protecting against adverse remodeling in HF, more basic work is needed to identify which of the signaling cascades or others are critical for the protective effect. Although one could question whether creatine deficiency per se induces an insult, prior studies reported unchanged mitochondrial organization, compartmentation of high-energy phosphates and more than 500 proteins, including those of the respiratory complexes in creatine-deficient GAMT$^{-/-}$ as compared to wildtype mouse hearts.[64,65] Thus the loss of protection from adverse remodeling in CKmito hearts crossed with GAMT$^{-/-}$ creatine-deficient mice is best attributed to reduced ADP regeneration capacity rather than a potential insult not identified in several prior studies. Similar studies of loss of protection from adverse remodeling were not performed in CKmyofib hearts crossed with GAMT$^{-/-}$ mice since CKmyofib overexpression does not protect from pathologic remodeling (Figure 2N,O) like CKmito (Figure 2J,K). CKmito increases certain basal antioxidant activities and survival in response to H_2O_2 exposure and this may indicate that CKmito overexpression induces a stress response that may also contribute to benefit. We note that normality testing may not have been sufficiently powered for the smaller data sets and thus we provide both parametric and nonparametric testing in the data supplement for those data sets.

In summary, pathologic hypertrophy and adverse remodeling are well-established, clinically important predictors of adverse outcomes in patients with HF. Abnormalities in energy metabolism occur in the failing heart that were previously associated with contractile abnormalities, but the role, if any, of altered energy metabolism in human HF remodeling was less well characterized. The present observations are the first to identify a close relationship between pathologic hypertrophy and adverse remodeling with reduced cardiac ATP levels and synthesis through CK in the failing human heart. Moreover, it is not the cytosolic myofibrillar CK isoform, but the mitochondrial CK isoform, sitting at the intersection of cardiac mitochondrial ADP regeneration-ATP transfer and pathologic ROS generation, that plays a critical role in mitigating pathologic hypertrophy and adverse remodeling in the failing heart. Thus, mitochondrial CK offers a potential new target for improving depressed metabolism, reducing ROS and their consequences, and limiting adverse remodeling in heart failure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Sources of Funding:

Non-Standard Abbreviations:

ADP	adenosine diphosphate
Akt	protein kinase B
ATP	adenosine triphosphate
CK	creatine kinase
CKmyofib	myofibrillar isoform of creatine kinase
CKmito	mitochondrial isoform of creatine kinase
c-Myc	protooncogene c-Myc
EDV	end-diastolic volume
EF	ejection fraction
EPR	electron paramagnetic resonance
FOXO3	forkhead box O3
GAMT$^{-/-}$ mice	Guanidinoaceate N-metthyltransferase knock-out mice
GATA-4	transcription factor GATA-4

HF	heart failure
ISO	isoproterenol
LV	left ventricle
LVH	left ventricular hypertrophy
MRI	magnetic resonance imaging
MRS	magnetic resonance spectroscopy
PCr	creatine phosphate
ROS	reactive oxygen species
TAC	transverse aortic constriction
TrxR	thioredoxin reductase

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Novelty and Significance:

What is known?

- Heart failure is characterized by abnormalities in contraction/relaxation and pathologic ventricular remodeling, whereby the heart wall hypertrophies (thickens) and eventually dilates. The latter has been associated with adverse heart failure outcomes and increased mortality in heart failure patients.

- Impaired cardiac energy metabolism occurs in heart failure and is closely related to contractile abnormalities. However, the role of impaired energy metabolism in heart failure-associated pathologic remodeling is not known.

What new information does this article contribute?

- In patients with heart failure, pathologic ventricular hypertrophy and dilatation are closely associated with low myocardial ATP levels and reduced rates of ATP synthesis through creatine kinase, the primary energy reserve reaction.

- In mouse models of hypertrophy and heart failure, rescuing the mitochondrial isoform of creatine kinase, rather than the cytosolic myofibrillar isoform, significantly limits the development of pathologic hypertrophy and adverse remodeling.

- The ability of mitochondrial creatine kinase, sitting at the crossroads of myocardial energy production and redox balance, to regenerate ADP for mitochondrial ATP synthesis is critical for this protection from pathologic remodeling.

- The findings demonstrate that metabolic strategies and interventions to improve mitochondrial health and mitochondrial creatine kinase activity are critical players in reducing pathologic remodeling in heart failure and the closely linked consequences of adverse clinical outcomes and increased mortality.

Cardiac mitochondrial energy metabolism is impaired in heart failure (HF), but its role in HF-related pathologic remodeling is poorly understood. Creatine kinase (CK), the primary muscle energy reserve reaction which rapidly provides ATP at the myofibrils (via a myofibrillar isoform, CKmyofib) and regenerates mitochondrial ADP (via a mitochondrial isoform, CKmito), is down-regulated in HF. We measured in vivo cardiac energetics and remodeling in people and observed that pathologic LV hypertrophy and dilatation correlate closely with reduced ATP levels and rates of ATP synthesis through CK. Metabolic rescue with cardiac-specific overexpression of either CKmyofib or CKmito was studied in mice for its impact on maladaptive hypertrophy. TAC-induced LV hypertrophy and dilatation were attenuated by overexpression of CKmito, but not by CKmyofib. CKmito overexpression also attenuated isoproterenol-induced hypertrophy. When the CK reaction was limited by creatine substrate depletion, protection was lost in CKmito mice, indicating ADP regeneration and not the protein per se is critical in limiting adverse HF remodeling. In human HF, pathologic hypertrophy and adverse

remodeling are closely related to ATP levels and CK energy reaction deficits. Sitting at the intersection of cardiac energetics and redox balance, CK mito plays a crucial role in attenuating pathologic remodeling in HF.
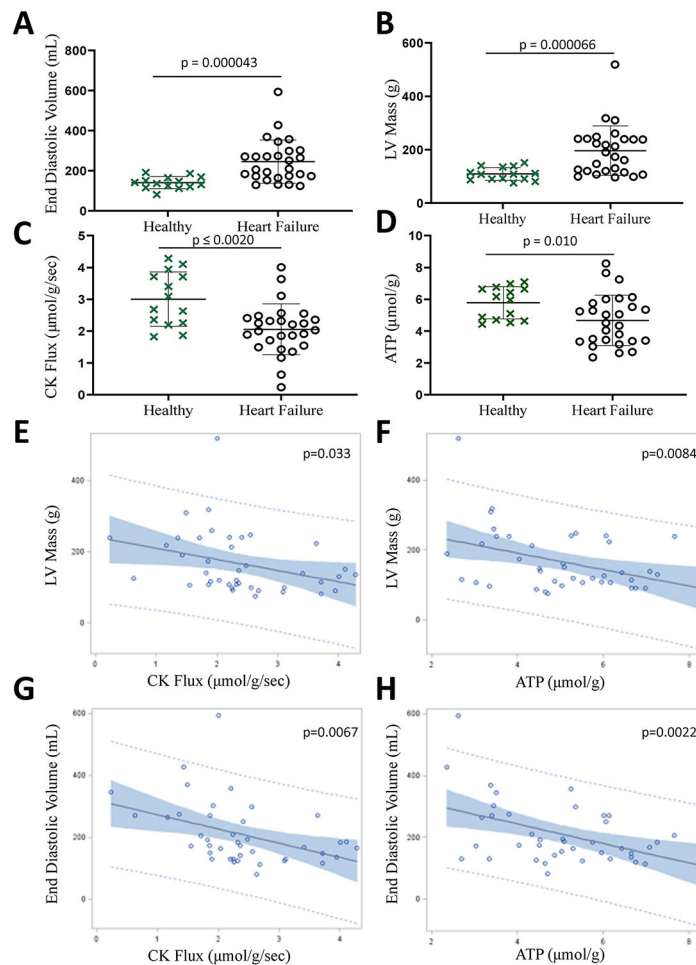
Figure 1. Pathologic hypertrophy and adverse remodeling are associated with decreased cardiac energy metabolism in HF patients.

Cardiac end-diastolic volume (**A**) and left ventricular (LV) mass (**B**) measured by in vivo MRI were higher, and cardiac CK flux (**C**) and ATP levels (**D**) measured by 31P MRS were lower in HF patients as compared to healthy subjects. Pathologic remodeling was associated with energy deficits as noted by correlations between LV mass and CK flux (**E,** p=0.033); LV mass and ATP levels (**F**, p=0.0084); end-diastolic volume and CK flux (**G**, p=0.0067); and end-diastolic volume and ATP levels (**H**, p=0.0022). Graphs show data points for individual participants. Data were tested for normality using the Kolmogorov-Smirnov test for normality and analyzed by two-tailed Student's *t*-test (**A-D**) or a linear regression model (**E-H**) (n = 14 (healthy controls), 27 (heart failure patients). The error bars represent ±SEM.
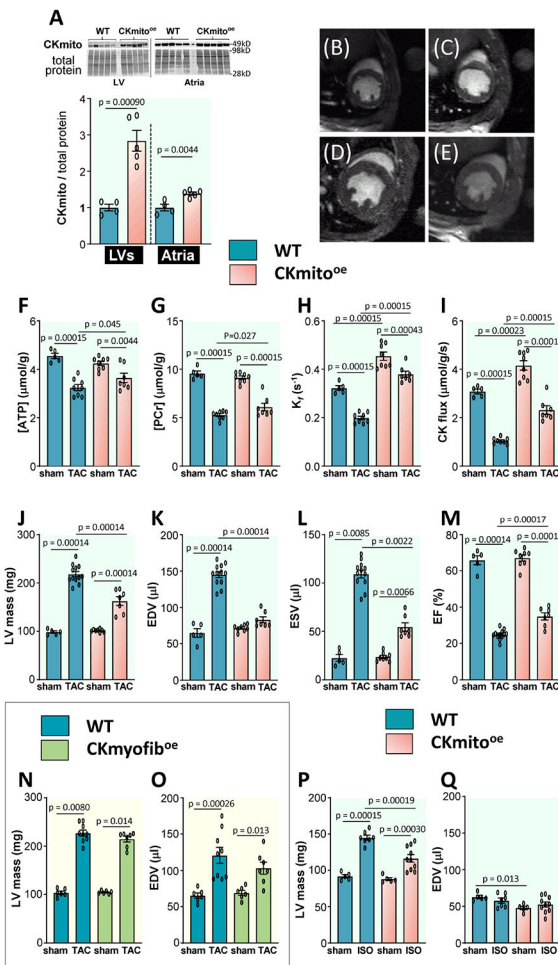
Figure 2. Cardiac-specific overexpression of mitochondrial creatine kinase (CKmito) improves *in vivo* cardiac energetics and attenuates pathologic remodeling in failing murine hearts.
Tissues were isolated from WT and cardiac-specific CKmito overexpressing (CKmito[oe]) mice. **(A)** Expression levels of CKmito were normalized to total protein and presented as relative to the amount of CKmito detected in WT hearts (experimental replicates: n=4 (WT LV or atria), n=5 (CKmito LV or atria)). Representative transverse *in vivo* [1]H MRI of mice at the mid-left ventricle showing **(B)** WT sham, **(C)** CKmito[oe] sham, **(D)** WT TAC, and **(E)** CKmito[oe] TAC. In vivo cardiac ^{31}P MRS and MRI measures of **(F)** ATP and **(G)** phosphocreatine (PCr) levels; **(H)** CK forward pseudo-first-order rate constant (K_f); **(I)** CK flux; **(J)** left ventricular (LV) mass; **(K)** end-diastolic volume (EDV); **(L)** end-systolic volume (ESV); and **(M)** ejection fraction (EF) for WT or CKmito[oe] mice with or without TAC (experimental replicates: n=5 (WT sham), n=8 (WT TAC or CKmito[oe] sham), and n=7 (CKmito[oe] TAC) for **F-I** and n=5 (WT sham), n=12 (WT TAC), n=8 (CKmito[oe] sham), and n=7 (CKmito[oe] TAC) for **J-M**), **(N)** LV mass and **(O)** EDV for WT or cardiac-specific myofibrillar CK overexpressing (CKmyofib[oe]) mice with or without TAC (experimental replicates: n=6 (WT sham or CKmyofib[oe] sham), n=9 (WT TAC), n=7 (CKmyofib[oe] TAC)); and **(P)** LV mass, and **(Q)** EDV for WT or CKmito[oe] mice following chronic isoproterenol (ISO) or saline infusion (experimental replicates: n=5 (WT saline or CKmito[oe] saline), n=7 (WT ISO), and n=10 (CKmito[oe] ISO)). Graphs show data points for individual mice. Data

were tested for normality using the Kolmogorov-Smirnov test for normality and analyzed by two-tailed Student's *t*-test **(A)**, two-way ANOVA followed by Tukey's post-hoc multiple comparison tests **(F-K, M, O-Q),** or Wilcoxon signed rank test followed by pair-wise, two-sided multiple comparison analysis (Dwass, Steel, Crichlow-Fligner Method) **(L, N)**. The error bars represent ±SEM.
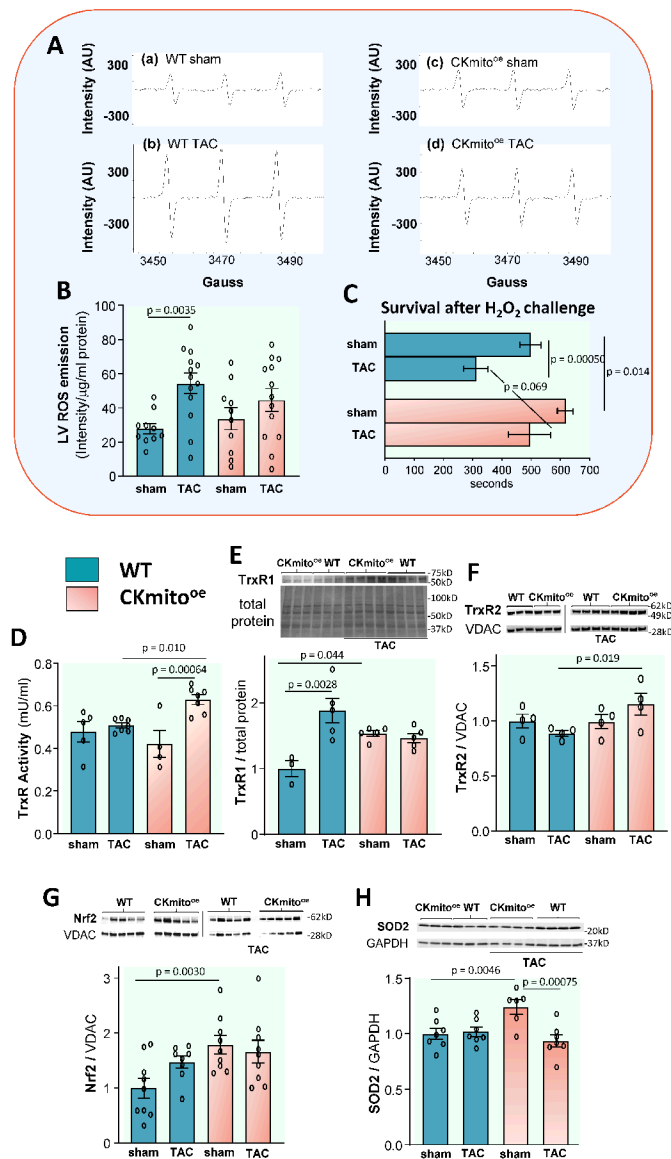
Figure 3. Cardiac-specific overexpression of mitochondrial creatine kinase (CKmito) attenuates reactive oxygen species (ROS) due to TAC heart failure.

(**A**) Representative electron paramagnetic resonance (EPR) spectra showing oxygen-derived free radicals detected in left ventricles (LV) from WT (**a**) sham or (**b**) TAC hearts, and LVs from cardiac-specific CKmito overexpressing (CKmito[oe]) (**c**) sham or (**d**) TAC hearts. (**B**) ROS production measured by EPR in LVs of WT or CKmito[oe] sham or TAC hearts (experimental replicates: n=10 (WT sham or CKmito[oe] sham) and n=13 (WT TAC or CKmito[oe] TAC)). (**C**) Cardiomyocytes isolated from WT[‡] or CKmito[oe] sham or TAC hearts were exposed to H_2O_2 (50 μM) for 700 s, and the time to irreversible arrhythmia/cell death was monitored (experimental replicates: n=20 cells isolated from 6 mice (WT sham), n=6 cells isolated from 2 mice (WT TAC), n=20 cells isolated from 5 mice (CKmito[oe] sham), and n=10 cells isolated from 4 mice (CKmito[oe] TAC)). Note that the same WT cardiomyocytes were employed for Fig 3C and Supplement Figure S4E since the

experiments were conducted on the same day and under the same experimental conditions. **(D)** Thioredoxin reductase (TrxR) activities determined in WT or CKmito^{oe} sham or TAC heart LV homogenates in the presence of excess NADPH (experimental replicates: n=5 (WT sham), n=7 (WT TAC or CKmito TAC), and n=4 (CKmito sham)). Representative immunoblots and summary of data showing LV expression levels of: **(E)** TrxR1; **(F)** TrxR2; **(G)** Nrf2 (nuclear factor erythroid 2-related factor 2); and **(H)** SOD2 (superoxide dismutase 2) normalized to total protein, GAPDH (glyceraldehyde 3-phosphate dehydrogenase), or VDAC (voltage-dependent anion channel) and presented as relative to the amount of protein detected in sham WT hearts (experimental replicates: n=3 (WT sham) and n=5 (WT TAC, CKmito sham, or CKmito TAC) **(E)**; n=4 per group **(F)**; n=9 (WT sham, CKmito sham, or CKmito TAC) and n=8 (WT TAC) **(G)**; n=7 (WT sham, WT TAC, or CKmito TAC) and n=6 (CKmito sham) **(H)**. Graphs show data points for individual mice **(B, D-H)**. Data were tested for normality using the Kolmogorov-Smirnov test and analyzed by two-way ANOVA followed by Tukey's post-hoc multiple comparison test **(B, D-H)** while a generalized estimating equation model was used to take into account the correlation of within-subject data **(C)**. The error bars represent ±SEM.
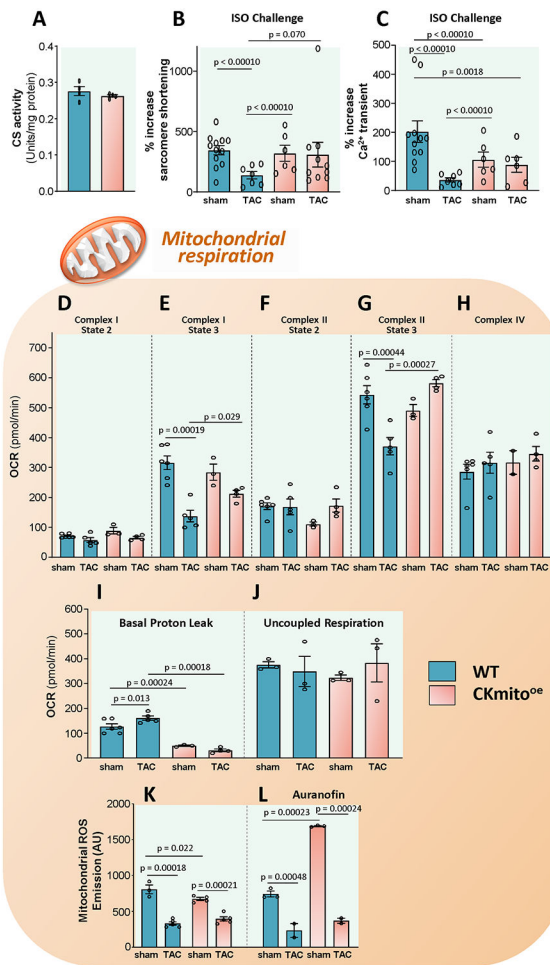
Figure 4. Cardiac-specific overexpression of mitochondrial creatine kinase (CKmito) improves isolated mitochondrial function and reduces proton leak.

(**A**) Citrate synthase (CS) activities, an index of total mitochondrial mass, analyzed in WT, and cardiac-specific CKmito overexpressing (CKmito[oe]) heart lysates (n=4 per group). Cardiomyocytes isolated from WT or CKmito[oe] sham or TAC hearts were stimulated with isoproterenol (ISO, 2.5 nM, 10 min) and percent increase in: (**B**) sarcomere shortening and (**C**) Ca^{2+} transients were monitored (experimental replicates: n=12 cells isolated from 3 mice (WT sham), n=7 cells isolated from 2 mice (WT TAC), n=6 cells isolated from 2 mice (CKmito[oe] sham), and n=10 cells isolated from 6 mice (CKmito[oe] TAC) cells (**B**) and n=11 cells isolated from 3 mice (WT sham), n=7 cells isolated from 2 mice (WT TAC), n=6 cells isolated from 2 mice (CKmito[oe] sham), and n=6 cells isolated from 2 mice (CKmito[oe] TAC) (**C**)). Mitochondria were isolated from WT and CKmito[oe] sham or TAC hearts. Summary of data showing oxygen consumption rate (OCR) for: (**D**) complex I state 2; (**E**) complex I state 3; (**F**) complex II state 2; (**G**) complex II state 3; and (**H**) complex IV; (**I**) basal proton leak; and (**J**) uncoupled respiration (experimental replicates: n=6 (WT sham), n=5 (WT TAC), n=3 (CKmito sham), and n=4 (CKmito TAC) (**D-G, I**); n=6 (WT sham), n=5 (WT TAC), n=2 (CKmito sham), and n=4 (CKmito TAC) (**H**); n=3 per group (**J**)). Mitochondrial reactive oxygen species (ROS) emission in the (**K**) absence or (**L**) presence of auranofin (20nM) (experimental replicates: n=3 (WT sham), n=5 (WT TAC or CKmito TAC), and n=4

(CKmito sham) **(K)**; n=3 (WT sham or CKmito sham) and n=2 (WT TAC or CKmito TAC) **(L)**). Graphs show data points for individual mice **(A, D-L)** or individual cells **(B, C)**. Data were tested for normality using the Kolmogorov-Smirnov test for normality and analyzed using a two-tailed Student's *t*-test **(A),** a generalized estimating equation model was used to take into account the correlation of within-subject data **(B, C)**, or two-way ANOVA followed by Tukey's post-hoc multiple comparison test **(D-L)**. The error bars represent ±SEM.
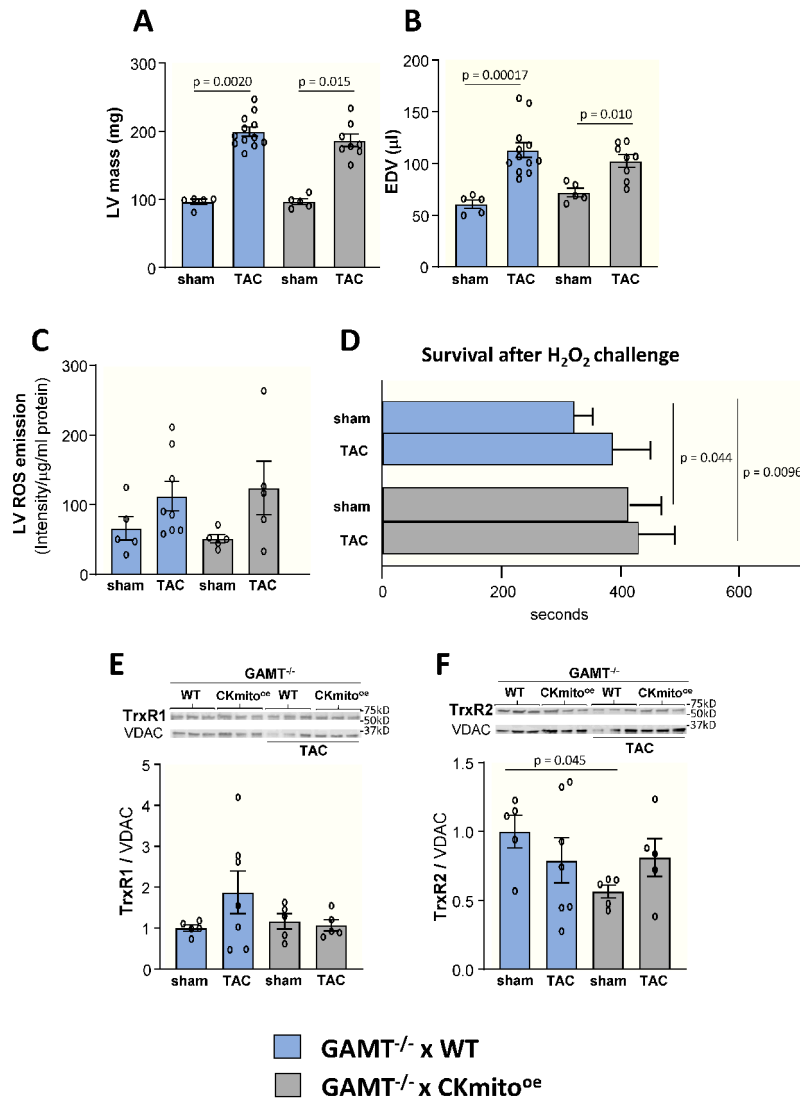
Figure 5. Cardiac-specific overexpression of mitochondrial creatine kinase (CKmito) does not attenuate pathologic remodeling and ROS burden in creatine-deficient mice.

Creatine-deficient guanidinoacetate N-methyltransferase (GAMT) knock-out mice were crossed with cardiac-specific CKmito overexpressing (GAMT$^{-/-}$ x CKmito^{oe}) or WT (GAMT$^{-/-}$ x WT) mice. Cardiac **(A)** left ventricular (LV) mass and **(B)** end-diastolic volume (EDV) for GAMT$^{-/-}$ x CKmito^{oe} and GAMT$^{-/-}$ x WT mice with or without TAC were determined by *in vivo* MRI (experimental replicates: n=5 (GAMT$^{-/-}$ x WT sham or GAMT$^{-/-}$ x CKmito^{oe} sham), n=12 (GAMT$^{-/-}$ x WT TAC), and n=8 (GAMT$^{-/-}$ x CKmito^{oe} TAC) **(A-B)**. **(C)** Reactive oxygen species (ROS) production measured by EPR spectroscopy in LVs of GAMT$^{-/-}$ x CKmito^{oe} and GAMT$^{-/-}$ x WT sham or TAC hearts (experimental replicates: n=5 (GAMT$^{-/-}$ x WT sham, GAMT$^{-/-}$ x CKmito^{oe} sham, GAMT$^{-/-}$ x CKmito^{oe} TAC) and n=8 (GAMT$^{-/-}$ x WT TAC). **(D)** Cardiomyocytes were isolated from GAMT$^{-/-}$ x CKmito^{oe}, and GAMT$^{-/-}$ x WT sham or TAC hearts were exposed to H$_2$O$_2$ (50 μM) for 700 s and time to irreversible arrhythmia/cell death was monitored (experimental replicates: n=19 cells isolated from 3 mice (GAMT$^{-/-}$ x

WT sham), n=12 cells isolated from 2 mice (GAMT$^{-/-}$ x WT TAC or GAMT$^{-/-}$ x CKmito^{oe} TAC), and n=18 cells isolated from 3 mice (GAMT$^{-/-}$ x CKmito^{oe} sham)). Representative immunoblots and summary of data showing LV expression levels of **(E)** TrxR1 (thioredoxin reductase 1) and **(F)** TrxR2 (thioredoxin reductase 2) normalized to VDAC (voltage-dependent anion channel) and presented as relative to the amount of protein detected in sham GAMT$^{-/-}$ x WT hearts (experimental replicates: n=5 (GAMT$^{-/-}$ x WT sham, GAMT$^{-/-}$ x CKmito^{oe} sham, GAMT$^{-/-}$ x CKmito^{oe} TAC) and n=7 (GAMT$^{-/-}$ x WT TAC) **(E-F)**). Graphs show data points for individual mice **(A-C, E-F)**. Data were tested for normality using the Kolmogorov-Smirnov test for normality and analyzed by Wilcoxon signed rank test followed by pair-wise, two-sided multiple comparison analysis (Dwass, Steel, Crichlow-Fligner Method) **(A)**, two-way ANOVA followed by Tukey's post-hoc multiple comparison test **(B-C,E-F)**, or a generalized estimating equation model was used to take into account the correlation of within-subject data. **(D)**. The error bars represent ±SEM.
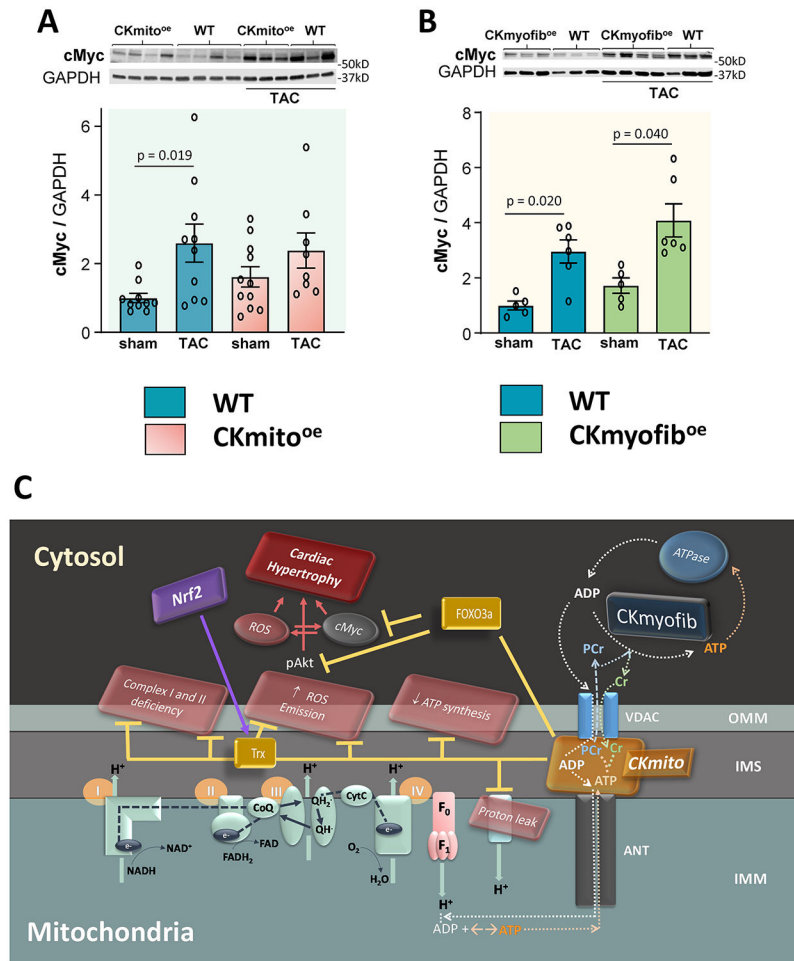
Figure 6. Overexpression of mitochondrial creatine kinase (CKmito) maintains pro-/anti-hypertrophic transcription factor balance in TAC failing hearts.

Left ventricular (LV) tissue from WT, cardiac-specific CKmito overexpressing (CKmito^oe), or cardiac-specific myofibrillar CK overexpressing (CKmyofib^oe) sham or TAC hearts was isolated and analyzed. Representative immunoblots and summary of data showing expression levels of cMyc (Myc protooncogene protein) in: WT, CKmito^oe **(A)**; or CKmyofib^oe **(B)** sham or TAC hearts normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and presented as relative to the amount of protein detected in sham WT hearts (experimental replicates: n=10 (WT sham or WT TAC), n=11 (CKmito^oe sham), and n=8 (CKmito^oe TAC) **(A)**; n=5 (WT sham or CKmyofib^oe sham) and n=6 (WT TAC or CKmyofib^oe TAC) **(B)**. Data were tested for normality using the Kolmogorov-Smirnov test for normality and analyzed by non-parametric Kruskal-Wallis test, followed by pair-wise, two-sided multiple comparison analysis (Dunn method with Benjamini-Hochberg adjustment). The error bars represent ±SEM. **(C)** Scheme summarizing the attenuation of cardiac hypertrophy and dysfunction during pressure overload in CKmito^oe hearts. Abbreviations: OMM (outer mitochondrial membrane), IMM (inner mitochondrial membrane), Trx (thioredoxin), ROS (reactive oxygen species), Nrf2 (nuclear factor erythroid 2-related factor 2), CoQ (coenzyme Q), QH• (semiquinone), QH$_2$• (ubiquinol), CytC (cytochrome C), F$_1$F$_0$ (F$_1$F$_0$-ATP synthase), ANT (adenine nucleotide translocator), VDAC

(voltage-dependent anion channel), CKmyofib (myofibrillar creatine kinase), CKmito (mitochondrial creatine kinase)