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Activation of Serine One-Carbon Metabolism by Calcineurin Aβ1 Reduces Myocardial Hypertrophy and Improves Ventricular Function

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ABSTRACT

BACKGROUND In response to pressure overload, the heart develops ventricular hypertrophy that progressively decompensates and leads to heart failure. This pathological hypertrophy is mediated, among others, by the phosphatase calcineurin and is characterized by metabolic changes that impair energy production by mitochondria.

OBJECTIVES The authors aimed to determine the role of the calcineurin splicing variant CnAβ1 in the context of cardiac hypertrophy and its mechanism of action.

METHODS Transgenic mice overexpressing CnAβ1 specifically in cardiomyocytes and mice lacking the unique C-terminal domain in CnAβ1 (*CnAβ1*$^{\Delta i12}$ mice) were used. Pressure overload hypertrophy was induced by transaortic constriction. Cardiac function was measured by echocardiography. Mice were characterized using various molecular analyses.

RESULTS In contrast to other calcineurin isoforms, the authors show here that cardiac-specific overexpression of CnAβ1 in transgenic mice reduces cardiac hypertrophy and improves cardiac function. This effect is mediated by activation of serine and one-carbon metabolism, and the production of antioxidant mediators that prevent mitochondrial protein oxidation and preserve ATP production. The induction of enzymes involved in this metabolic pathway by CnAβ1 is dependent on mTOR activity. Inhibition of serine and one-carbon metabolism blocks the beneficial effects of CnAβ1. *CnAβ1*$^{\Delta i12}$ mice show increased cardiac hypertrophy and declined contractility.

CONCLUSIONS The metabolic reprogramming induced by CnAβ1 redefines the role of calcineurin in the heart and shows for the first time that activation of the serine and one-carbon pathway has beneficial effects on cardiac hypertrophy and function, paving the way for new therapeutic approaches. (J Am Coll Cardiol 2018;71:654–67)

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Cardiac hypertrophy progressively decompensates and becomes maladaptive, leading to pathological cardiac remodeling and heart failure (1). Maladaptive cardiac hypertrophy is accompanied by interstitial and perivascular fibrosis and by changes in cardiac metabolism. The hypertrophic heart progressively reverts to an embryonic metabolic program with reduced fatty acid oxidation and increased reliance on glucose metabolism that result in decreased ATP production (2).

The calcium-regulated phosphatase calcineurin plays a major role in pathological hypertrophy. Calcineurin is composed of a catalytic (CnA) and a regulatory (CnB) subunit. CnA is encoded by 3 different genes (resulting in CnAα, CnAβ, and CnAγ), with CnAβ being the main isoform in the heart. Two splice variants for CnAβ have been described. Although CnAβ2 has a C-terminal autoinhibitory domain and acts like a typical CnA, CnAβ1 has a unique C-terminal domain, not shared by any other known protein, that confers these isoform specific properties (3-6).

Constitutive activation of calcineurin or its main target, the transcription factor nuclear factor of activated T cells (NFATc), leads to massive maladaptive cardiac hypertrophy (7). By contrast, mice lacking CnAβ show reduced ventricular hypertrophy in response to pressure overload (8). However, the role of CnAβ1 in this context is unknown.

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METHODS

Full Methods can be found in the Online Appendix.

MICE. *αMHC-CnAβ1* mice express the human CnAβ1 isoform in a cardiomyocyte-restricted manner under the control of the alpha myosin heavy chain (αMHC) promoter (4). CnAβ1$^{\Delta i12}$ mice were generated by deleting intron 12-13 in the gene that encodes CnAβ (*Ppp3cb*), which encodes the unique C-terminal domain in CnAβ1. Only adult male mice were used in this study. All procedures were approved by the ethics committees of the CNIC and the Regional Government of Madrid.

SURGERIES AND ECHOCARDIOGRAPHIC ANALYSIS. Maladaptive cardiac hypertrophy was induced by transaortic constriction (TAC) trying to reproduce the human condition as much as possible (9). Where indicated, L-buthionine-sulfoximine (BSO) (3 g/l in drinking water) or NCT-503 (0.9 mg/mouse, daily intraperitoneal injection) was administered for 21 days, starting on the day of the surgery. Transthoracic echocardiography was performed blindly using an ultra-high-resolution echocardiography system with a linear 30-MHz transducer. Two-dimensional and M-mode echocardiography in parasternal long- and short-axis views were performed blinded as previously described and recorded for posterior blinded analysis (3).

RESULTS

CnAβ1 OVEREXPRESSION REDUCES CARDIAC HYPERTROPHY. To determine the effect of CnAβ1 overexpression on the heart in the context of maladaptive cardiac hypertrophy, we used *αMHC-CnAβ1* transgenic mice that overexpress CnAβ1 in a cardiac-specific manner (Online Figures 1A and 1B) (4). We induced pressure overload in wild-type (WT) and transgenic mice by TAC, and we analyzed cardiac function 21 days later. Transgenic mice showed a significantly reduced heart weight to body weight ratio after TAC compared with WT mice (**Figure 1A**). Similarly, echocardiographic analysis revealed a reduced left ventricular mass index (LVMi) and thinner posterior wall and interventricular septum in CnAβ1-overexpressing mice after TAC compared with WT mice (**Figures 1B–1D**). In agreement with the echocardiography results, transgenic mice showed a more limited increase in cardiomyocyte size (**Figure 1E**). Importantly, whereas contractility declined in WT mice 21 days after TAC, it was preserved in CnAβ1-overexpressing mice, as shown by improved left ventricular ejection fraction (LVEF) (**Figure 1F**). Transgenic mice also showed a limited increase in the expression of the HF markers atrial natriuretic factor (ANF) and brain natriuretic peptide (BNP), which were strongly induced in WT mice (Online Figures 1C and 1D).

Pressure overload hypertrophy was characterized by both interstitial and perivascular fibrosis in WT mice (**Figures 1G to 1J**). Transgenic mice showed significantly reduced cardiac fibrosis with levels similar to those of sham-operated mice. In agreement

ABBREVIATIONS AND ACRONYMS

ATF4 = activating transcription factor 4

BSO = L-buthionine-sulfoximine

GSH = reduced glutathione

LVEF = left ventricular ejection fraction

LVMi = left ventricular mass index

mTOR = mechanistic target of rapamycin

NFAT = nuclear factor of activated T cells

qRT-PCR = quantitative reverse-transcription real-time polymerase chain reaction

TAC = transaortic constriction

WT = wild-type

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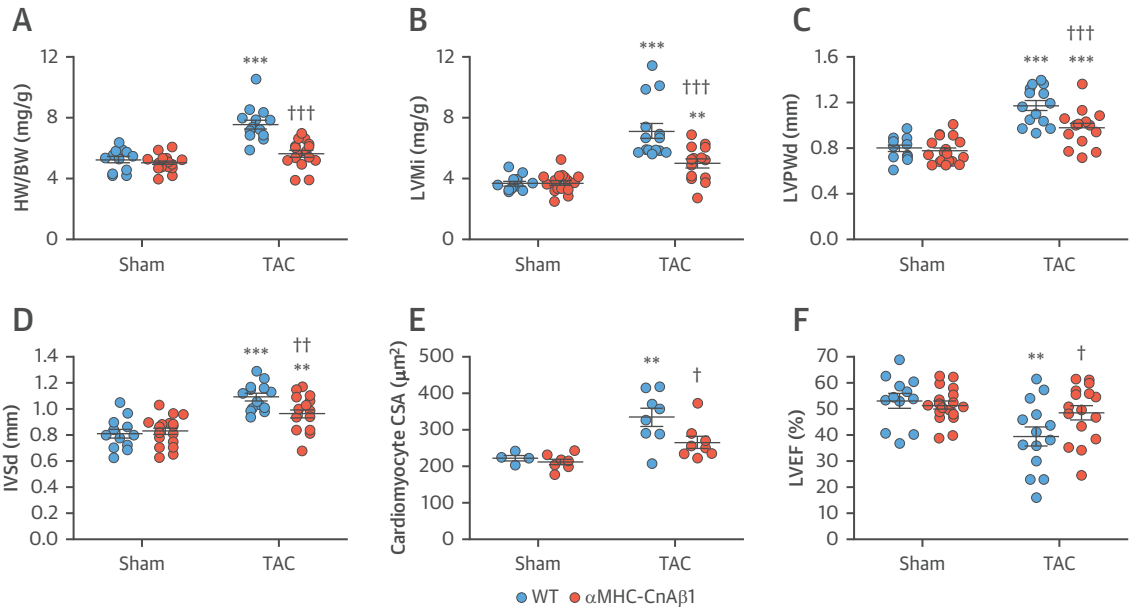
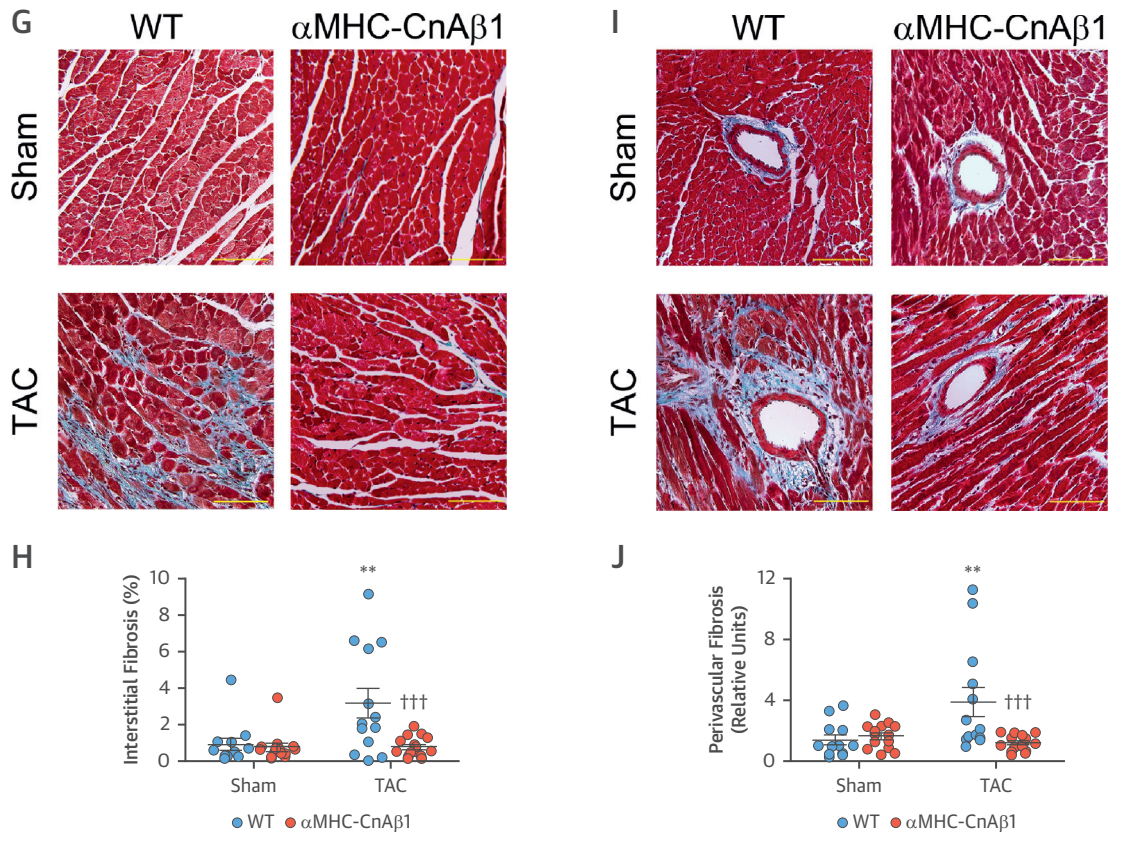
FIGURE 1 **CnAβ1 Overexpression Reduces Cardiac Hypertrophy**





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with these results, we also observed reduced expression of the fibrosis markers collagen Iα1 (Col1a1) and lysyl oxidase (Lox) in CnAβ1-overexpressing mice, together with reduced levels of SMAD2 phosphorylation, suggesting reduced TGF-β signaling (Online Figures 1E to 1G).

To confirm the functional improvement elicited by CnAβ1 in a second transgenic mouse line, we used rtTA-CnAβ1 mice in which CnAβ1 expression is induced ~3-fold upon treatment with doxycycline (3). We found that CnAβ1 overexpression significantly reduced the left ventricular mass and improved systolic function 42 days after TAC, confirming the results obtained in *αMHC-CnAβ1* mice (Online Figure 2). Together, these results demonstrate that CnAβ1, unlike other calcineurin isoforms, reduces maladaptive hypertrophy and cardiac remodeling following pressure overload.

CnAβ1 OVEREXPRESSION DOES NOT INTERFERE WITH NFAT ACTIVATION. To determine whether CnAβ1 overexpression interferes with the activation of the endogenous NFAT pathway, we first quantified the expression of Rcan1 isoforms. TAC induced a significant increase of the Rcan1.4 isoform, which is strongly regulated by NFAT, but not the Rcan1.1 isoform. No difference in the expression of Rcan1.4 was observed between WT and *αMHC-CnAβ1* mice (**Figures 2A and 2B**). To confirm the activation of the NFAT pathway, we crossed WT and *αMHC-CnAβ1* mice with a reporter mouse line in which the expression of luciferase is controlled by NFAT binding sites. A strong NFAT activation was observed in both WT and transgenic mice in response to pressure overload, as determined by luciferase activity, without any substantial change between both mouse types (**Figure 2C**). These results indicate that CnAβ1 overexpression does not interfere with NFAT activation in cardiac hypertrophy.

CnAβ1 ACTIVATES SERINE AND ONE-CARBON METABOLISM. We next analyzed the transcription profile of WT and transgenic mice using microarrays. Following TAC, WT mice showed a consistent up-regulation of genes associated with extracellular matrix, cell adhesion, and fibrosis (Online Tables 1 and 2) and a down-regulation of genes associated with the mitochondria (Online Tables 3 and 4).

Interestingly, sham *αMHC-CnAβ1* mice showed a strong up-regulation of the genes involved in serine synthesis, phosphoglycerate dehydrogenase (Phgdh), and phosphoserine aminotransferase 1 (Psat1), and in the one-carbon pathway, including methyltetrahydrofolate dehydrogenase 2 (Mthfd2) and aldehyde dehydrogenase 1 L2 (Aldh1l2) (**Figure 2D,** Online Tables 5 to 8). Using quantitative reverse-transcription real-time polymerase chain reaction (qRT-PCR), we confirmed the significant induction of Phgdh, Psat1, Shmt2, Mthfd2, and Aldh1l2 genes in *αMHC-CnAβ1* mice, after both sham and TAC surgery (**Figures 2E to 2I**). In addition, most of these genes, as well as CnAβ1 itself, showed smaller, but significant, expression changes in WT mice following TAC (Online Figure 3), suggesting that endogenous CnAβ1 and the serine and one-carbon pathway may play a role in the response to pressure overload.

Although no gene categories changed significantly in transgenic mice after TAC (Online Tables 9 to 12), *αMHC-CnAβ1* mice showed reduced expression of extracellular-matrix-related genes compared with WT (Online Tables 13 to 16), which was confirmed by qRT-PCR (Online Figures 1E and 1F).

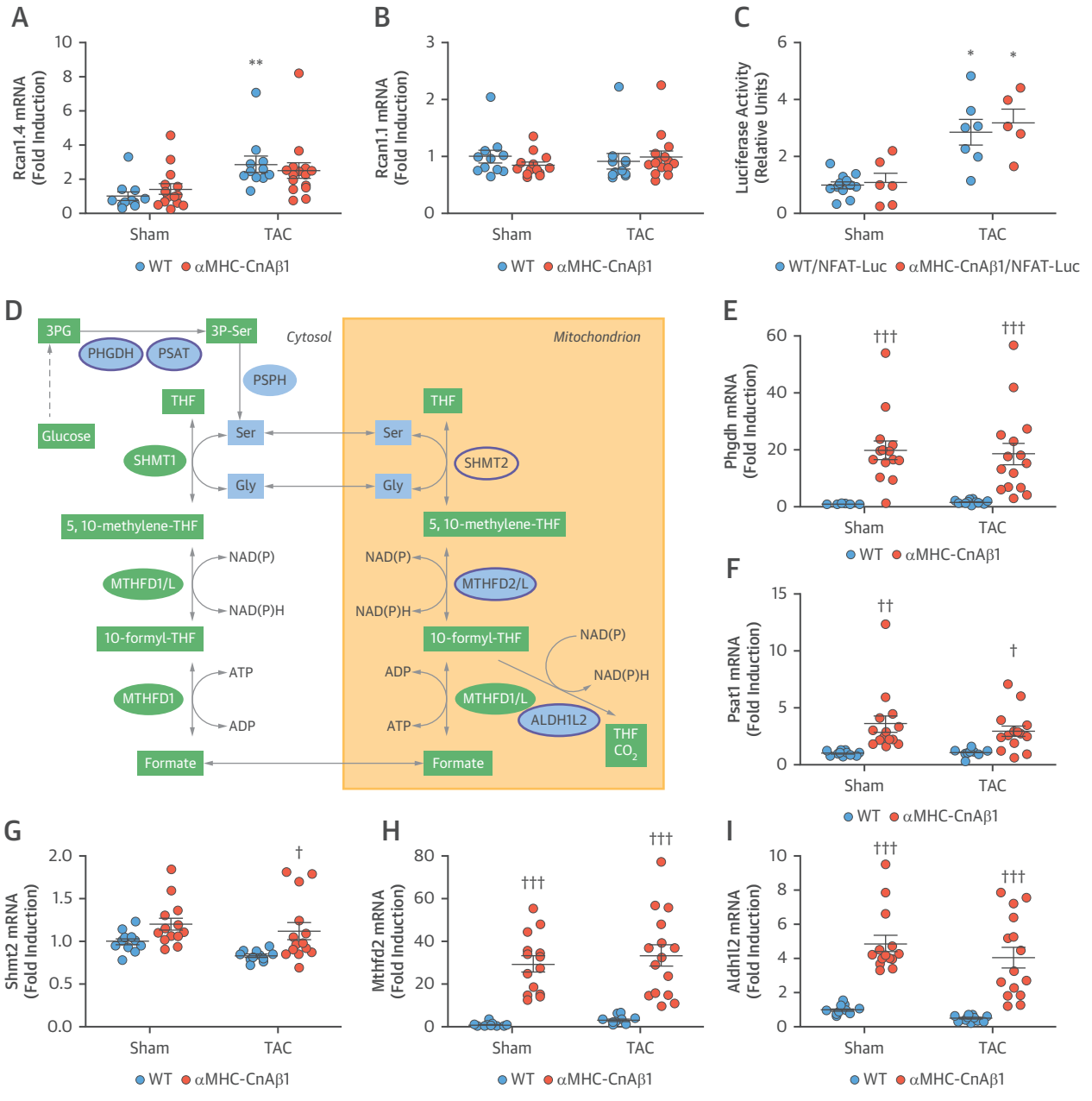
We then carried out a quantitative proteomics analysis of sham WT and transgenic mice, which also showed a strong increase in proteins involved in the serine and one-carbon pathway in *αMHC-CnAβ1* mice (Online Table 17, **Figure 2D**), confirming that serine and one-carbon metabolism was strongly induced in the heart by CnAβ1.

Many of these genes are regulated by the mTOR signaling pathway through the transcription factor activating transcription factor 4 (ATF4) (4,10). To determine whether this pathway was activated by CnAβ1 in the context of pressure overload, we analyzed these proteins by Western blot. We observed activation of the mTOR/AKT signaling pathway and increased expression of ATF4 in CnAβ1-overexpressing

FIGURE 1 Continued

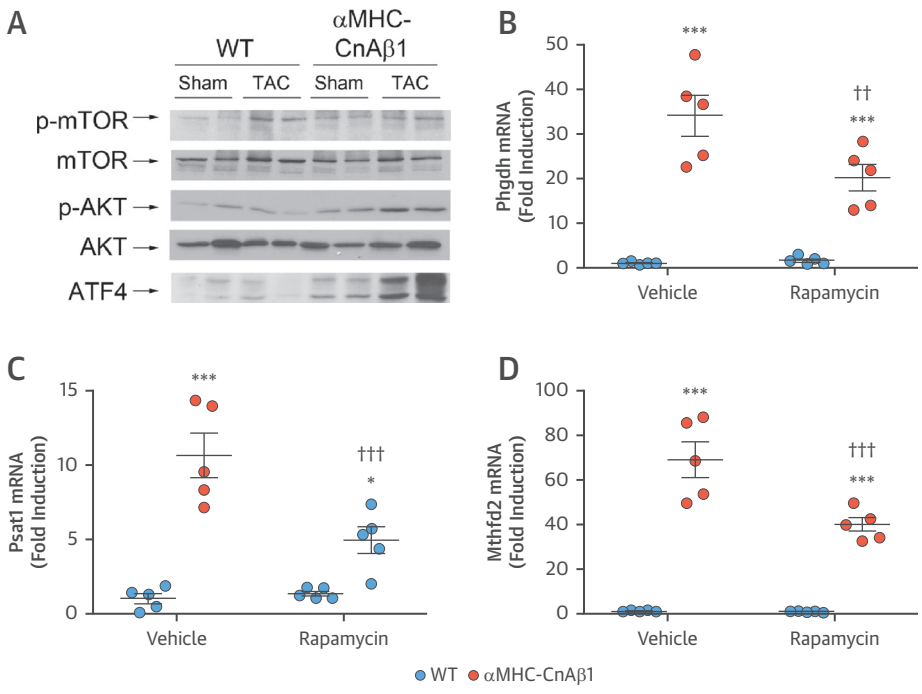
(A) Pressure overload cardiac hypertrophy was induced in wild type (WT) and *αMHC-CnAβ1* mice by transaortic constriction (TAC) and the heart weight to body weight ratio was determined 21 days after surgery, compared with sham-operated animals. LVMi **(B)**, LVPWd **(C)**, and IVSd **(D)** were determined by echocardiography 21 days after surgery. **(E)** Cardiomyocyte cross-sectional area was analyzed by immunohistochemistry. **(F)** LVEF was determined by echocardiography. Interstitial **(G and H)** and perivascular **(I and J)** cardiac fibrosis were analyzed 21 days after TAC using Masson's trichrome protocol. **Bar** indicates 100 μm. Interstitial fibrosis was quantified as the percentage of area in each picture that is stained as collagen. Perivascular fibrosis was calculated as the perivascular collagen area relative to the vessel area. At least 2 pictures per mouse were analyzed. Values represent average ± SEM. $^{**}p < 0.01$, $^{***}p < 0.005$ TAC versus sham for each mouse line; †p < 0.05, ††p < 0.01, †††p < 0.005 WT versus transgenic mice; 2-way analysis of variance plus Bonferroni post-test. n = 12 to 18. CSA = cross-sectional area; HW/BW = heart weight to body weight ratio; IVSd = interventricular septum thickness in diastole; LVEF = left ventricular ejection fraction; LVMi = left ventricular mass index; LVPWd = left ventricular posterior wall thickness in diastole.

FIGURE 2 CnAβ1 Activates the Serine and One-Carbon Metabolic Pathway Without Interfering With NFAT-Mediated Transcription



Expression of the Rcan1.4 **(A)** and Rcan1.1 **(B)** mRNA was analyzed by qRT-PCR 21 days after TAC. **(C)** WT and *αMHC-CnAβ1* mice were crossed with reporter mice in which luciferase expression is controlled by an NFAT binding site multimer. Luciferase activity normalized to total protein content was analyzed in heart extracts 21 days post-TAC. **(D)** Summary of genes involved in the serine and one-carbon pathway. Components induced in *αMHC-CnAβ1* mice are indicated by a **blue fill** (mRNA, metabolites) or **purple border** (protein). Expression of Phgdh **(E)**, Psat1 **(F)**, Shmt2 **(G)**, Mthfd2 **(H)**, and Aldh1l2 **(I)** was quantified as in **(A)**. Values express average ± SEM. *p < 0.05, **p < 0.01 TAC versus sham for each mouse line; †p < 0.05, ††p < 0.01, †††p < 0.005 WT versus transgenic mice; 2-way analysis of variance plus Bonferroni post-test. n = 10 to 15 **(A, B, and E–I)**; n = 5 to 12 **(C)**. 3PG = 3-phosphoglycerate; 3P-Ser = 3-phosphoserine; Gly = glycine; NAD(P) = nicotinamide adenine dinucleotide phosphate; NAD(P)H = nicotinamide adenine dinucleotide phosphate (reduced form); NFAT = nuclear factor of activated T cells; qRT-PCR = quantitative reverse-transcription real-time polymerase chain reaction; Ser = serine; THF = tetrahydrofolate; other abbreviations as in Figure 1.

FIGURE 3 mTOR Mediates the Activation of the Serine One-Carbon Pathway by CnAβ1



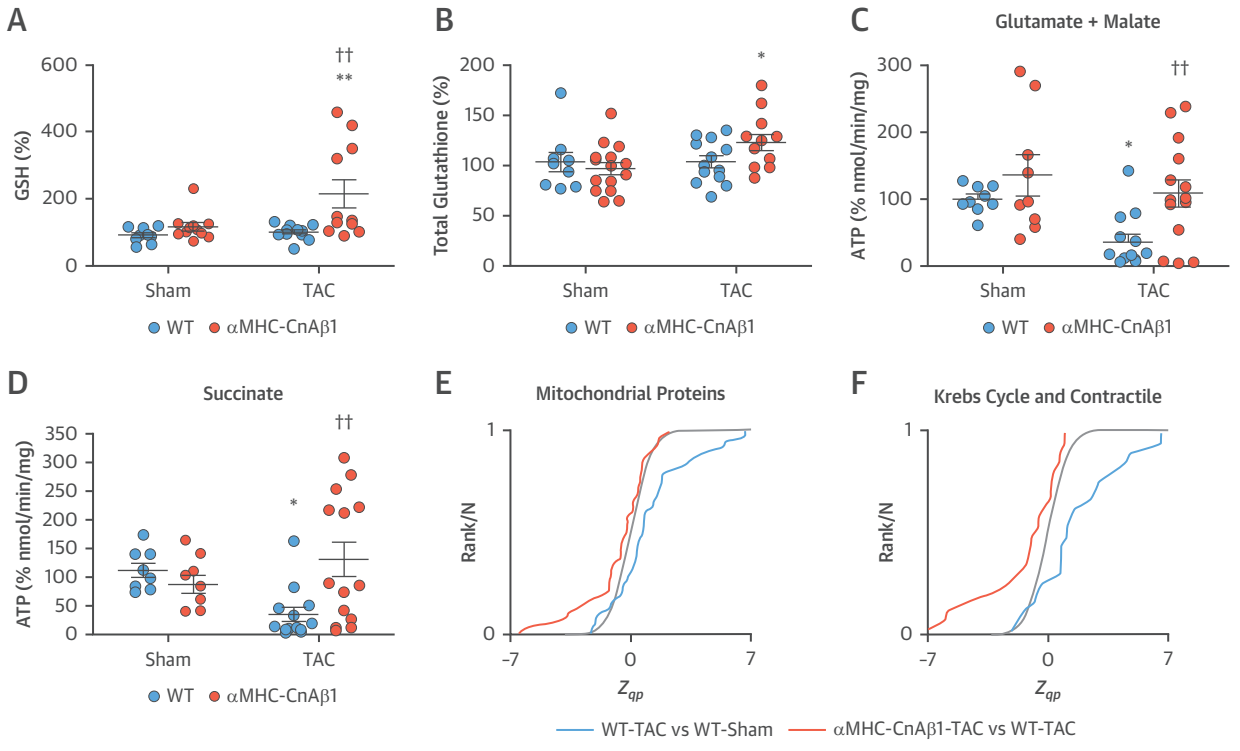
(A) Phosphorylation and/or expression of mTOR, Akt, and ATF4 was analyzed by Western blot in myocardial samples from the indicated mice 21 days after surgery. **(B to D)** WT and *αMHC-CnAβ1* uninjured mice were treated with rapamycin for 5 days, and the expression of Phgdh, Psat1, and Mthfd2 was analyzed in myocardial samples. *p < 0.05, ***p < 0.005 *αMHC-CnAβ1* versus WT; ††p < 0.01, †††p < 0.005 rapamycin versus vehicle; 2-way analysis of variance plus Bonferroni post-test. n = 5. ATF4 = activating transcription factor 4; mTOR = mechanistic target of rapamycin; other abbreviations as in Figure 1.

mice (Figure 3A), consistent with our previous results (3,4). To investigate whether mTOR mediates the induction of the serine and one-carbon pathway by CnAβ1, we treated uninjured WT and transgenic mice with the mTOR inhibitor rapamycin, and we analyzed the mice 5 days later. As shown in Figures 3B to 3D, mTOR inhibition partially abolished the induction of Phgdh, Psat1, and Mthfd2 in *αMHC-CnAβ1* mice, indicating that CnAβ1 activates the serine and one-carbon pathway through mTOR signaling.

CnAβ1 OVEREXPRESSION CHANGES THE HEART'S METABOLOME. Phdgh-derived serine synthesis and the one-carbon cycle supply metabolites for the production of antioxidant mediators such as glutathione (11,12). A thorough metabolomic analysis in uninjured WT and *αMHC-CnAβ1* mice detected a total of 462 different metabolites in myocardial samples from both mouse types, with 133 of them being up-regulated in transgenic mice and 65 metabolites down-regulated (Online Table 18). A principal component analysis showed strong separation of the samples, suggesting a different metabolic profile in WT and CnAβ1-overexpressing mice (Online Figure 4A). Using Metabolomic Pathway Analysis, we found a strong enrichment of metabolites related to glutathione, amino acid, and nucleotide metabolic pathways (Online Figure 4B).

To explore these changes more in depth, we analyzed the amount of different metabolites belonging to the affected pathways. We found a strong significant induction of glutathione, mainly reduced glutathione (GSH), and its precursor metabolites (Online Figures 4C and 5, Online Table 18). This was accompanied by a significant induction of methionine metabolites (Online Figure 4D). In addition, *αMHC-CnAβ1* mice showed a significant increase of several metabolites associated with polyamine metabolism and the urea cycle, mainly related to the degradation of arginine to ornithine, putrescine, and spermidine (Online Figure 4E, Online Table 18). We also found that several metabolites related to purine and pyrimidine metabolism were up-regulated, leading to a strong induction of the potent antioxidant urate (Online Table 18). These changes were

FIGURE 4 CnAβ1 Reduces the Oxidative Damage After Pressure Overload and Improves ATP Synthesis



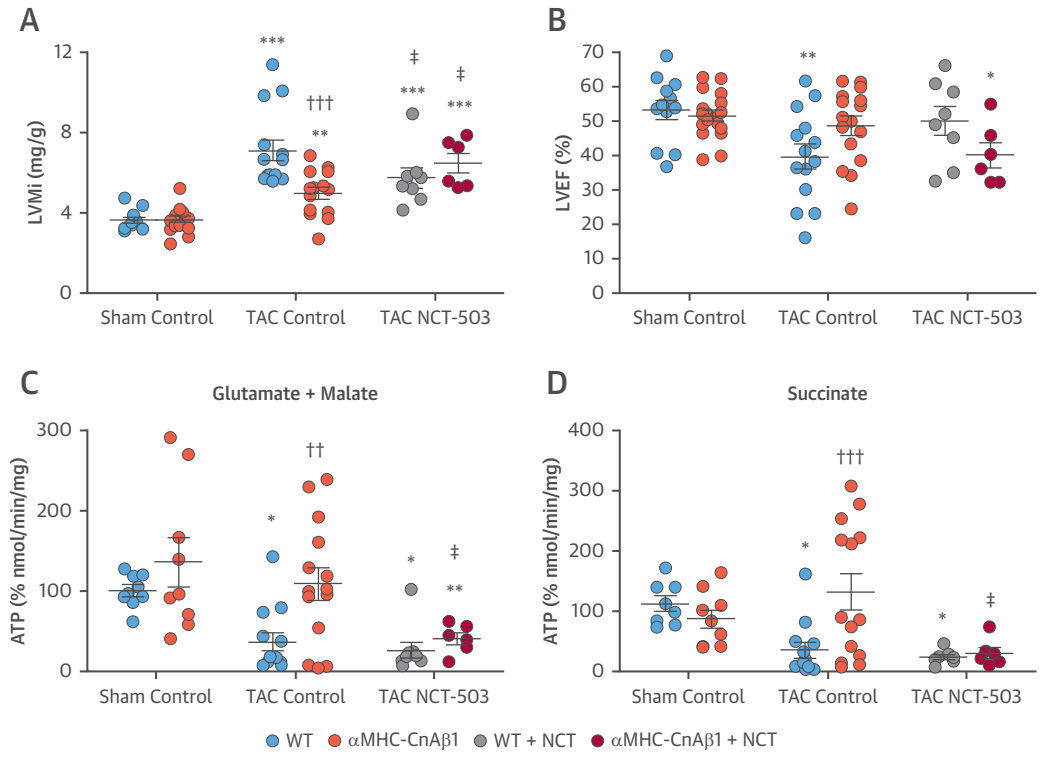
Reduced **(A)** and total **(B)** glutathione, and ATP generation driven by glutamate + malate **(C)** or by succinate **(D)** were quantified in whole myocardium from WT and *αMHC-CnAβ1* mice 21 days after TAC or sham surgery. *p < 0.05, **p < 0.01 versus sham; ††p < 0.01 WT versus transgenic mice; 2-way analysis of variance plus Bonferroni post-test. n = 9 to 15 **(A and B)**, n = 8 to 14 **(C and D)**. **(E and F)** Quantitative redox proteomics analysis using the GELSILOX technique. Shown are the cumulative distributions of the standardized $\log_2$ ratios of abundances (Z_{qp}) of peptides containing oxidized cysteine residues in the conditions that are compared. The peptides belong to either mitochondrial proteins **(E)** or proteins associated with the Krebs cycle or muscle contraction **(F)**. The null hypothesis distribution, which reflects identical peptide abundances in the 2 conditions, is represented by a **gray line**. The **blue lines** show the relative abundance of peptides from WT mice after TAC in relation to those from WT mice after sham surgery, whereas the **orange lines** represent the peptides from αMHC-CnAβ1 mice after TAC in relation to WT mice after TAC. Positive values of Z_{qp} indicate increase in relative peptide abundance. ATP = adenosine triphosphate; GSH = reduced glutathione; other abbreviations as in **Figure 1**.

paralleled by a strong increase in another antioxidant, ascorbate (Online Figure 4F). Together, these changes indicated that CnAβ1 activates several metabolic pathways with antioxidant activity.

MITOCHONDRIAL COMPLEXES SHOW INCREASED ACTIVITY IN CnAβ1-OVEREXPRESSING MICE. To determine whether the changes in the metabolic profile observed in transgenic mice would impact mitochondrial number or activity, we analyzed different parameters in uninjured mice. We first quantified mitochondrial DNA and found no significant differences between WT and *αMHC-CnAβ1* mice, although a small increase was observed in transgenic mice (Online Figure 6A). Analysis of mitochondrial complexes and supercomplexes in isolated mitochondria revealed no differences between both mouse types (Online Figure 6B). Similarly, we observed no obvious differences in the expression of individual complex components by Western blot (Online Figure 6C). Analysis of the activity of the different mitochondrial complexes showed a significant increase in the activity of complexes I+III and II+III in *αMHC-CnAβ1* mice (Online Figure 6D). In addition, we observed increased citrate synthase activity in transgenic mice, in agreement with the increase in citrate detected by mass spectrometry (Online Table 18) and with the slight increase in mtDNA copy number (mitochondrial mass). These changes in the activity of mitochondrial complexes, however, had no effect on the production of ATP by isolated mitochondria, neither driven by glutamate and malate nor by succinate (Online Figure 6E).

CnAβ1 REDUCES PROTEIN OXIDATION AND IMPROVES ATP SYNTHESIS FOLLOWING PRESSURE OVERLOAD. We next investigated whether the changes observed in uninjured mice were reproduced in the hypertrophic

FIGURE 5 Inhibition of Serine Synthesis From Glucose Abolishes the Beneficial Effects of CnAβ1

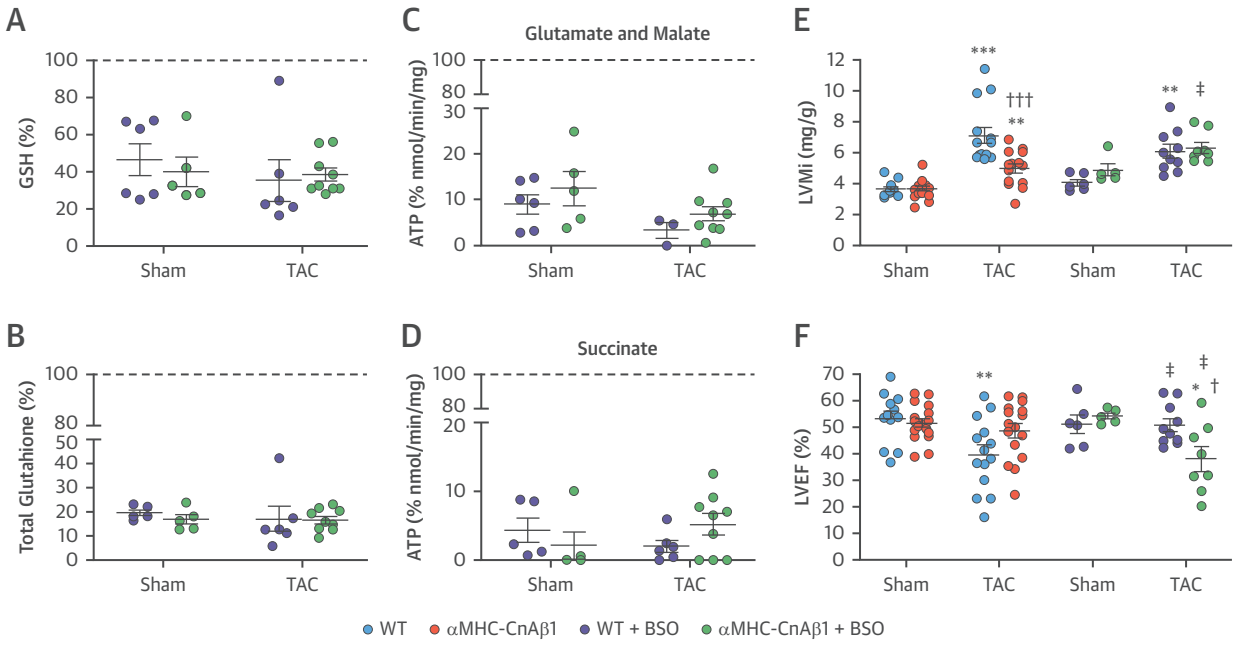


Mice underwent TAC or sham surgery and LVMi **(A)** and LVEF **(B)** were analyzed by echocardiography 21 days later. Where indicated, mice were treated with the PHGDH inhibitor NCT-503 for 21 days. ATP synthesis from glutamate and malate **(C)** or succinate **(D)** was analyzed in all mice. Note that the control (untreated) animals for these analyses are those shown in Figure 1 (for **A** and **B**) and Figure 6 (for **C** and **D**), and are repeated here for comparative purposes. *p < 0.05, **p < 0.01, ***p < 0.005 TAC versus sham for each mouse line; ††p < 0.01, †††p < 0.005 WT versus transgenic mice; ‡p < 0.05, TAC NCT-503–treated mice versus TAC untreated mice; 2-way analysis of variance plus Bonferroni post-test. n = 6 to 18 **(A and B)**, n = 6 to 14 **(C and D)**. Abbreviations as in Figures 1 and 4.

heart. Using an enzymatic assay, we found an increase in GSH in *αMHC-CnAβ1* mice compared with WT, which was more evident 21 days after TAC (Figure 4A). We also found an increase in total glutathione in transgenic mice, albeit at much lower levels (Figure 4B). We observed a strong reduction in the production of ATP by mitochondria isolated from WT mice after TAC, both driven by glutamate and malate (Figure 4C) and by succinate (Figure 4D). By contrast, mitochondria from *αMHC-CnAβ1* mice showed preserved ATP production level after TAC, significantly higher than that of WT mice (Figures 4C and 4D). Using the GELSILOX technique (13), we observed an increase in oxidized peptides from mitochondrial proteins, particularly those related to the Krebs cycle, and from muscle contraction proteins in WT mice 21 days after TAC (WT-TAC mice) (Figures 4E and 4F, Online Tables 19 to 21). Interestingly, *αMHC-CnAβ1* mice subjected to TAC showed reduced oxidation of the same peptide populations in comparison to WT-TAC mice (Figures 4E and 4F), suggesting that the antioxidant response elicited by CnAβ1 prevents mitochondrial and sarcomere protein oxidation, and hence improves ATP production and cardiac contraction.

ACTIVATION OF THE SERINE AND ONE-CARBON PATHWAY IS NECESSARY FOR THE INDUCTION OF ATP AND REDUCTION OF CARDIAC HYPERTROPHY BY CnAβ1. To determine the functional relevance of the activation of serine one-carbon metabolism by CnAβ1, we treated WT and transgenic mice for 21 days after TAC surgery with the PHGDH inhibitor NCT-503, which blocks the synthesis of serine from glucose and its contribution to the one-carbon pathway (14). Treatment of *αMHC-CnAβ1* mice with NCT-503 resulted in increased LVMi and reduced LVEF compared with untreated transgenic mice (Figures 5A and 5B). Furthermore, NCT-503 prevented the improved ATP

FIGURE 6 Glutathione Is Necessary for the Functional Improvement Induced by CnAβ1 After Pressure Overload



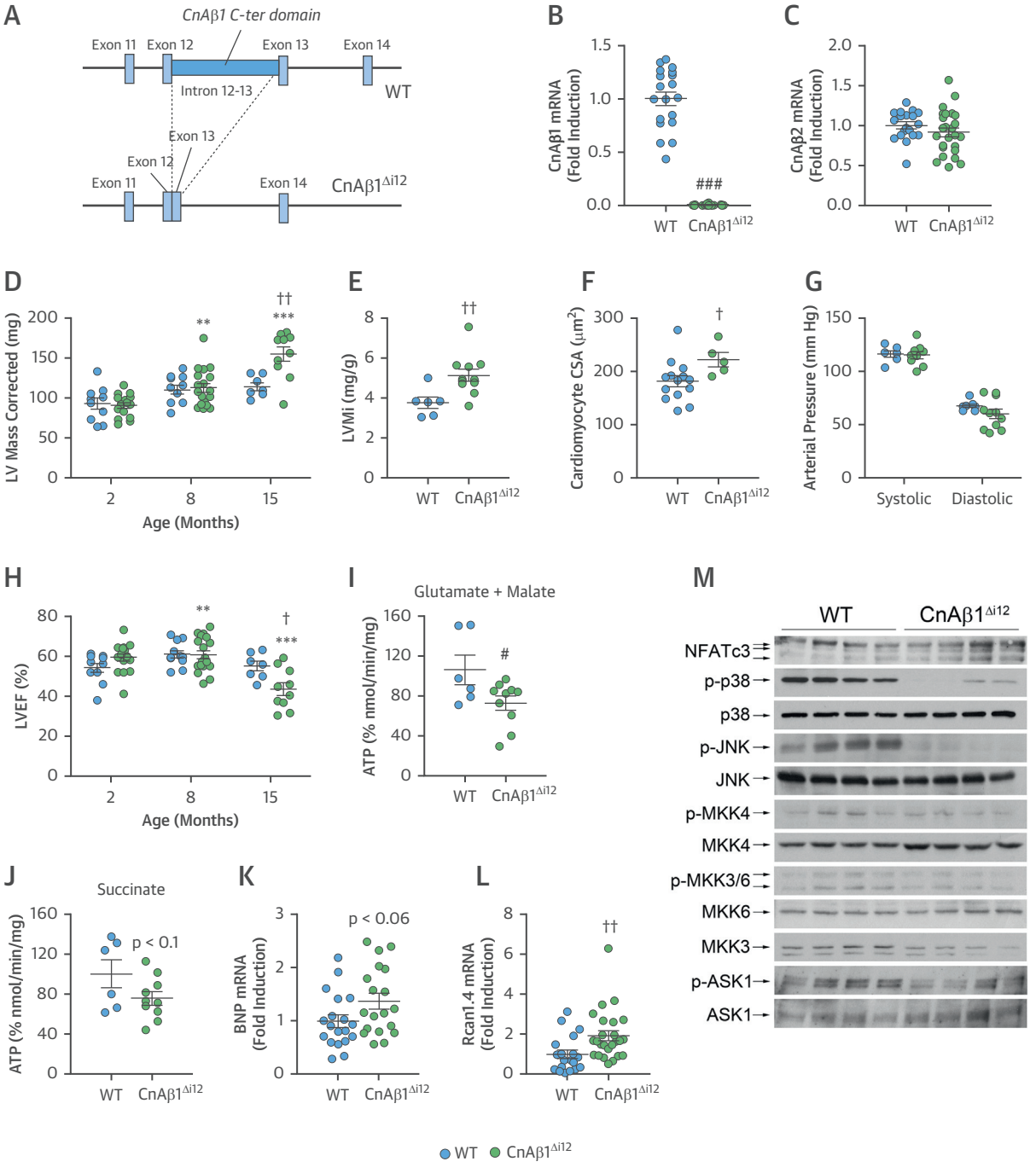
(A and B) WT and transgenic mice underwent sham or TAC surgery and were treated with L-buthionine-sulfoximine (BSO) for 21 days. Reduced **(A)** and total **(B)** glutathione, and ATP synthesis from glutamate and malate **(C)** or succinate **(D)** were measured in myocardial samples. Values are expressed as percentage ± SEM, referred to untreated WT sham mice **(dashed line)**. LVMi **(E)** and LVEF **(F)** were determined by echocardiography in BSO-treated and untreated (control) mice 21 days after surgery. Note that the control (untreated) animals for these comparisons are those shown in Figure 1, and are repeated here for comparative purposes. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, TAC versus sham for each mouse line; †$p < 0.05$, †††$p < 0.005$ *αMHC-CnAβ1* versus WT; ‡$p < 0.05$ BSO versus control; 2-way analysis of variance plus Bonferroni post-test. n = 5 to 9 **(A and B)**, n = 3 to 9 **(C and D)**, n = 5 to 18 **(E and F)**. Abbreviations as in Figures 1 and 4.

synthesis induced by CnAβ1, both from glutamate and malate, and from succinate. Transgenic mice showed similar ATP levels to those of WT mice and significantly reduced compared with untreated *αMHC-CnAβ1* mice (Figures 5C and 5D). These data indicate that the serine and one-carbon pathway is necessary for the beneficial effects of CnAβ1 on the heart.

GLUTATHIONE MEDIATES THE IMPROVED RESPONSE TO PRESSURE OVERLOAD IN *αMHC-CnAβ1* MICE. To find out whether the increase in reduced glutathione was also responsible for the improvement in ATP production and cardiac function observed in *αMHC-CnAβ1* mice after TAC, we treated both WT and CnAβ1 overexpressing mice with BSO, which decreases glutathione synthesis by inhibiting γ-glutamylcysteine synthetase (15). Administration of BSO resulted in a strong reduction in both GSH and total glutathione in both WT and transgenic mice, and prevented the increase in GSH induced by CnAβ1 (Figures 6A and 6B). The levels of untreated sham-operated WT mice are showed for reference. Inhibition of glutathione synthesis caused a strong decrease in ATP synthesis in all mice (Figures 6C and 6D), compared with untreated sham WT mice. This was accompanied by a slight reduction in ventricular wall thickness, although cardiac hypertrophy proceeded normally in response to TAC (Figure 6E). Importantly, in the presence of BSO, WT and *αMHC-CnAβ1* mice showed a similar increase in LVMi (Figure 6E). BSO treatment also resulted in reduced LVEF following TAC in CnAβ1-overexpressing mice, whereas no decline in cardiac contractility was observed in WT mice (Figure 6F). Together, these results suggest that the induction of GSH by CnAβ1 is necessary for the functional and structural improvement triggered by this calcineurin isoform and that in the absence of glutathione, the increased substrate flux through the oxidative phosphorylation chain induced by CnAβ1 may have deleterious effects on cardiac function decompensation following pressure overload.

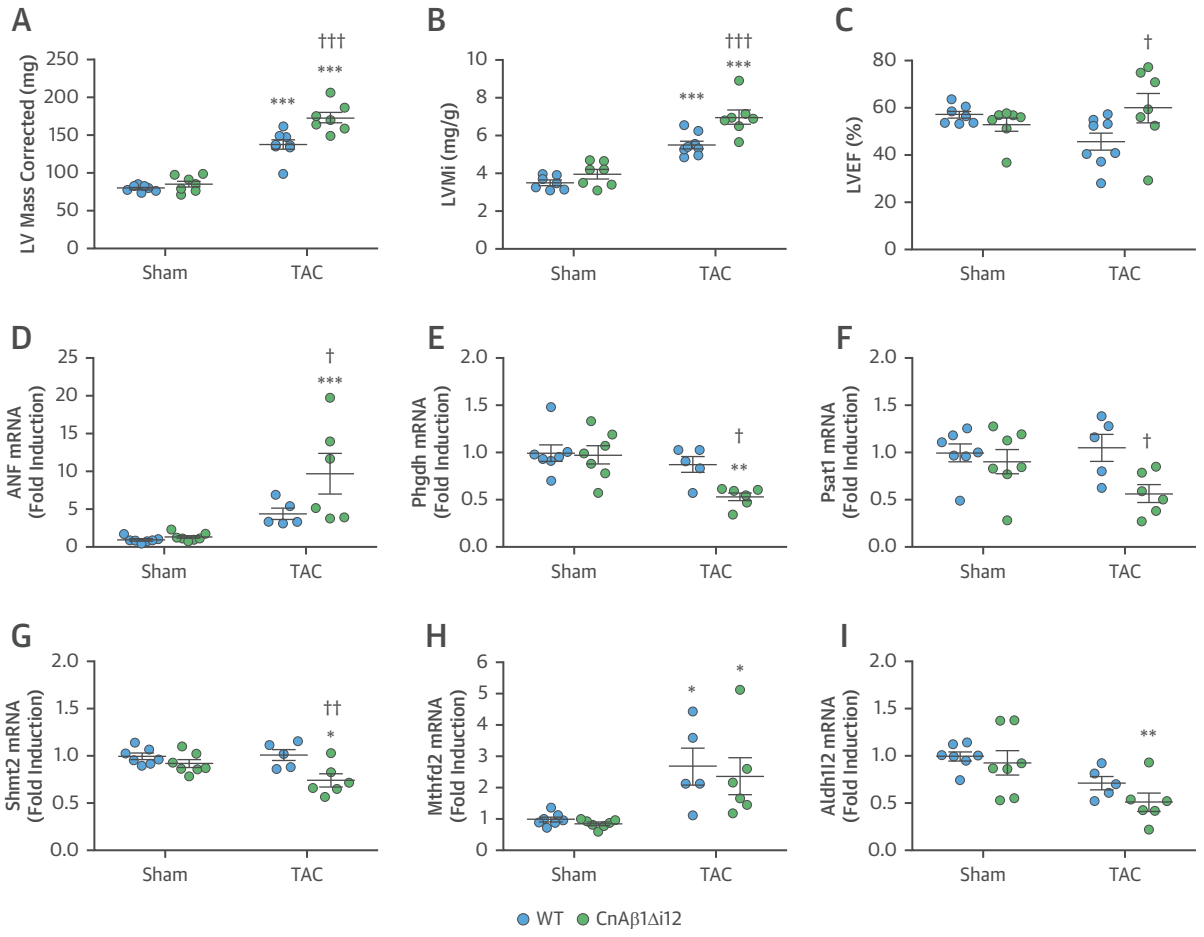
MICE LACKING CnAβ1 DEVELOP CARDIAC HYPERTROPHY. To determine the role of endogenous CnAβ1, we developed knockout mice lacking intron 12-13 in the CnAβ gene ($CnA\beta1^{\Delta i12}$ mice), which codes for the

FIGURE 7 Mice Lacking the C-Terminal Domain of CnAβ1 Show Increased Cardiac Hypertrophy



(A) Schematic of the strategy used to generate the *CnAβ*$^{\Delta i12}$ mice. **(B and C)** CnAβ1 and CnAβ2 expression was analyzed by qRT-PCR in WT and *CnAβ*$^{\Delta i12}$ mice. n = 19 to 25. The corrected **(D)** and indexed **(E)** left ventricular (LV) mass were analyzed by echocardiography at 2, 8, and 15 months of age. n = 6 to 18. **(F)** Cardiomyocyte cross-sectional area was analyzed by histological methods. n = 5 to 14. **(G)** Systolic and diastolic blood pressure were measured in 15-month-old WT and *CnAβ*$^{\Delta i12}$ mice. n = 6 to 11. **(H)** LVEF was analyzed by echocardiography at 2, 8, and 15 months. n = 7 to 18. ATP synthesis from glutamate and malate **(I)** or from succinate **(J)** was quantified in WT and *CnAβ*$^{\Delta i12}$ mice. n = 6 to 10. **(K and L)** BNP and Rcan1.4 mRNA expression was quantified by qRT-PCR. n = 19 to 25. **(M)** Western blot analysis of NFATc3 in WT and *CnAβ1*$^{\Delta i12}$ mice (n = 4) (smaller bands indicate activation by dephosphorylation). Phosphorylated (active) and total forms of kinases in the p38 and JNK pathways were also analyzed. **p < 0.01, ***p < 0.005, 8 and 15 months versus 2 months for each mouse line; †p < 0.05, ††p < 0.01, WT versus *CnAβ*$^{\Delta i12}$ mice; 2-way analysis of variance plus Bonferroni post-test. Abbreviations as in Figures 1 and 4.

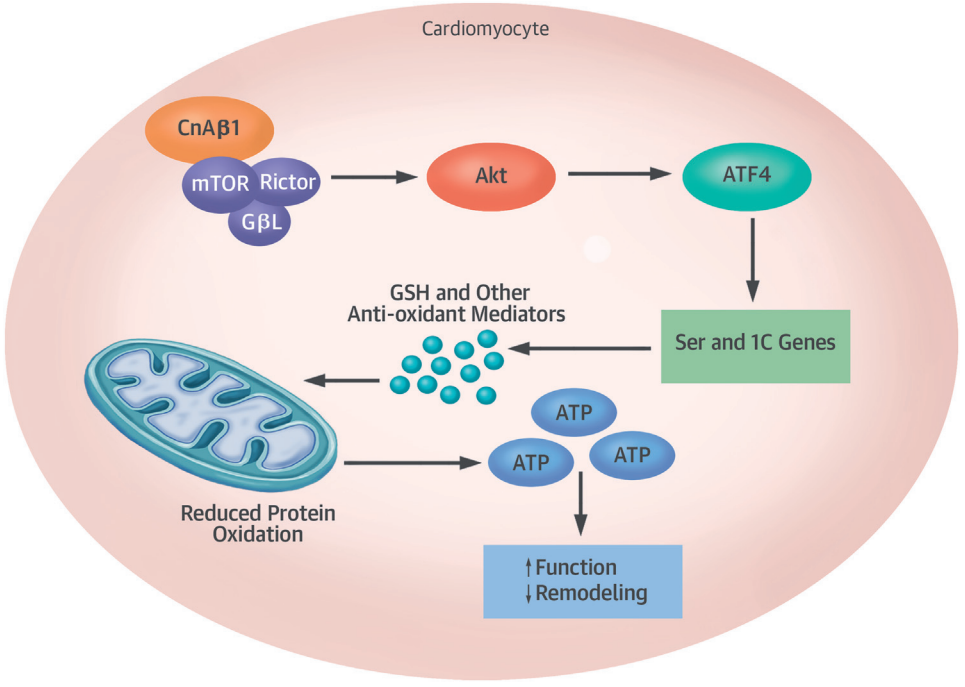
FIGURE 8 ***CnAβ$^{\Delta i12}$*** **Mice Develop Increased Hypertrophy Following Pressure Overload**



Left ventricular (LV) mass corrected **(A)**, LVMi **(B)**, and LVEF **(C)** were analyzed by echocardiography 21 days after TAC in WT and *CnAβ$^{\Delta i12}$* mice. n = 7 to 8. Expression of ANF **(D)**, Phgdh **(E)**, and Psat1 **(F)** was analyzed by WT and *CnAβ$^{\Delta i12}$* mice by qRT-PCR 21 days after surgery. n = 5 to 7. *p < 0.05, **p < 0.01, ***p < 0.005 TAC versus Sham for each mouse line; †p < 0.05, ††p < 0.01, †††p < 0.005, WT versus *CnAβ$^{\Delta i12}$* mice; 2-way analysis of variance plus Bonferroni post-test. ANF = atrial natriuretic factor; other abbreviations as in Figure 1.

unique C-terminal domain in CnAβ1 (Figure 7A). Although these mice express no CnAβ1, expression of the traditional isoform CnAβ2 is unaltered (Figures 7B and 7C). *CnAβ1$^{\Delta i12}$* mice are viable and fertile, and are born at the expected Mendelian ratio. However, we observed a significant increase in cardiac hypertrophy with age. Echocardiographic analysis showed increased left ventricular mass in *CnAβ1$^{\Delta i12}$* mice at 15 months of age and increased cardiomyocyte area (Figures 7D to 7F). No changes were observed in blood pressure (Figure 7G), suggesting that cardiac hypertrophy was not the consequence of systemic hypertension. Cardiac hypertrophy was accompanied by a decline in LVEF in CnAβ1-deficient mice (Figure 7H). This reduced contractile capacity was paralleled by a decreased ability to produce ATP, both from glutamate and malate and from succinate (Figures 7I and 7J). Furthermore, we found increased expression of BNP and the NFAT-regulated Rcan1.4 isoform in *CnAβ1$^{\Delta i12}$* mice (Figures 7K and 7L). Increased activation of NFATc3 was confirmed by Western blot (Figure 7M), and it was likely due to the inhibition of the MAPKs p38 and JNK, which rephosphorylate and inhibit NFATc. Inhibition of JNK and p38, their upstream kinases MKK4 and MKK3/6, or their upstream MKK kinase ASK1 has been reported to induce cardiac hypertrophy through NFAT activation (16-18). We found decreased phosphorylation (indicating decreased activation) of the MKK4/JNK and the

CENTRAL ILLUSTRATION Summary of CnAβ1 Signaling in the Cardiomyocyte and its Effect on Cardiac Function



Padrón-Barthe, L. et al. J Am Coll Cardiol. 2018;71(6):654–67.

CnAβ1 activates mTOR and ATF4 to induce serine and one-carbon metabolism and promote the production of GSH, among other mediators. Increased GSH results in reduced mitochondrial protein oxidation and increased ATP production, which improves cardiac function and remodeling after pressure overload. ATF4 = activating transcription factor 4; ATP = adenosine triphosphate; GβL = G protein beta subunit-like; GSH = reduced glutathione; mTOR = mechanistic target of rapamycin.

MKK3/6/p38 pathways and a mild inhibition of ASK1, which regulates both pathways, in $CnA\beta1^{\Delta i12}$ mice (Figure 7M). These results suggest that the increased hypertrophy observed in old $CnA\beta1^{\Delta i12}$ mice is the result of JNK and p38 inhibition and subsequent NFATc activation. We next studied the response of $CnA\beta1^{\Delta i12}$ mice to pressure overload. Three weeks after TAC, CnAβ1-deficient mice showed increased cardiac hypertrophy, indicated by the larger left ventricular mass (Figures 8A and 8B). LVEF, however, was higher in $CnA\beta1^{\Delta i12}$ than in control mice (Figure 8C), suggesting that cardiac hypertrophy had not yet decompensated. $CnA\beta1^{\Delta i12}$ mice showed higher ANF expression after TAC surgery, in agreement with the increased hypertrophy, and reduced expression of genes involved in serine and one-carbon metabolism, including Phgdh, Psat1, Shmt2, and Aldh1l2 (Figures 8D to 8I). These results suggest that CnAβ1 is necessary for normal energy homeostasis in the heart and that the disturbance of this pathway exacerbates cardiac hypertrophy.

DISCUSSION

Calcineurin activation plays a central role in the development of maladaptive cardiac hypertrophy. In contrast with this view, we show here that overexpression of the alternative splicing CnAβ variant CnAβ1 reduces cardiac hypertrophy and improves energy production, whereas deletion of its C-terminal unique domain results in spontaneous cardiac hypertrophy and decreased ATP synthesis, suggesting that the metabolic effects of calcineurin on the heart may be isoform-specific.

Interestingly, we found that CnAβ1 overexpression activates serine and one-carbon metabolism in an mTOR-dependent manner. This pathway is a major source of NADH and NADPH, and supplies metabolites to major metabolic pathways in the cells, including nucleotide biosynthesis, amino acid homeostasis, methylation reactions, and antioxidant defense (12,19). The serine and one-carbon pathway has recently gained strong attention in the cancer

research field (12). Many tumor cells are dependent on serine and increase Phgdh and Psat1 expression to increase serine availability, which provides these cells with a metabolic advantage (20). Serine and one-carbon metabolism is also triggered by mitochondrial dysfunction (21,22). Its role in the heart, however, is virtually unknown.

Here, we show for the first time to our knowledge that serine and one-carbon metabolism has a beneficial effect on the heart, necessary for the improved ATP synthesis and cardiac function induced by CnAβ1. Activation of this pathway leads to the generation of antioxidant metabolites that may be protective against reactive oxygen species, especially GSH. GSH production is reduced following pressure overload, and this reduction has a causal effect on the development of cardiac hypertrophy, fibrosis, and remodeling that leads to a decline in cardiac function after TAC (23). Consistent with this beneficial effect of GSH on the hypertrophic heart, we observed that inhibition of glutathione synthesis prevents the functional improvement induced by CnAβ1.

We found that NFAT is equally activated in WT and *αMHC-CnAβ1* mice after TAC, suggesting that CnAβ1 is not acting as an indirect inhibitor of NFAT. Instead, CnAβ1 activates the mechanistic target of rapamycin (mTOR)/Akt pathway and the transcription factor ATF4. We have previously reported that CnAβ1 activates this pathway by direct interaction of its C-terminal domain with the mTORC2 complex and that it induces ATF4 in an mTOR-dependent manner (4,6). Serine and one-carbon genes are known to be regulated by ATF4 upon activation of mTOR (10,11,21). These data, together with the inhibition of these genes by rapamycin in our transgenic mice, suggest that activation of the mTOR/ATF4 pathway by CnAβ1 induces metabolic changes that allow the cardiomyocyte to obtain energy and NADH/NADPH through the serine and one-carbon pathway, decreasing mitochondrial protein oxidation and preserving ATP production after pressure overload (**Central Illustration**).

We also report here the first description of mice lacking CnAβ1 following the deletion of CnAβ intron 12-13. $CnA\beta^{\Delta i12}$ mice are viable and fertile, and show no obvious malformation during embryonic development. However, they develop cardiac hypertrophy at 15 months of age. This is accompanied by a reduction in ATP production and a decline in cardiac function. In addition, we show that $CnA\beta^{\Delta i12}$ mice develop increased cardiac hypertrophy after TAC. We observed an improvement in LVEF in these mice, which was likely the direct result of hypertrophy itself, because cardiac function had not yet decompensated in these animals by 21 days. $CnA\beta^{\Delta i12}$ mice showed decreased expression of enzymes involved in the serine synthesis pathway, after either TAC or sham surgery, suggesting that activation of this pathway by CnAβ1 is necessary for the maintenance of energy balance and cardiac homeostasis.

STUDY LIMITATIONS. This study was performed in mice. Although we show evidence of the beneficial effects of CnAβ1 overexpression and activation of the serine and one-carbon pathway in the mouse heart, translation of results obtained in mice to humans is not always straightforward.

CONCLUSIONS

Our results demonstrate that CnAβ1 has an opposite effect on the heart to that of other calcineurin isoforms. We unveil the activation of the serine and one-carbon metabolic pathway by CnAβ1 and show its beneficial role in the heart for the first time. These changes in our understanding of cell signaling and metabolism during pressure overload hypertrophy may lead to the development of novel therapeutic avenues based on the modulation of serine and one-carbon metabolism.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: CnAβ1 overexpression in cardiomyocytes reduces left ventricular hypertrophy and remodeling, and improves systolic function following pressure overload. CnAβ1 promotes metabolic reprogramming of the myocardium, activating the serine one-carbon pathway, and generation of several antioxidant metabolites. These metabolic changes are necessary to preserve ATP synthesis and improve cardiac function in CnAβ1-overexpressing mice.

TRANSLATIONAL OUTLOOK: Further research is needed to explore the therapeutic potential of serine one-carbon pathway activation for patients with heart failure.

REFERENCES

1. Schiattarella GG, Hill JA. Inhibition of hypertrophy is a good therapeutic strategy in ventricular pressure overload. Circulation 2015;131:1435–47.

2. Doenst T, Nguyen TD, Abel ED. Cardiac metabolism in heart failure: implications beyond ATP production. Circ Res 2013;113:709–24.

3. López-Olañeta MM, Villalba M, Gómez-Salinero JM, et al. Induction of the calcineurin variant CnAβ1 after myocardial infarction reduces post-infarction ventricular remodelling by promoting infarct vascularization. Cardiovasc Res 2014;102:396–406.

4. Felkin LE, Narita T, Germack R, et al. Calcineurin splicing variant CnAβ1 improves cardiac function after myocardial infarction without inducing hypertrophy. Circulation 2011;123:2838–47.

5. Lara-Pezzi E, Winn N, Paul A, et al. A naturally occurring calcineurin variant inhibits FoxO activity and enhances skeletal muscle regeneration. J Cell Biol 2007;179:1205–18.

6. Gómez-Salinero JM, López-Olañeta MM, Ortiz-Sánchez P, et al. The calcineurin variant CnAβ1 controls mouse embryonic stem cell differentiation by directing mTORC2 membrane localization and activation. Cell Chem Biol 2016;23:1372–82.

7. Molkentin JD, Lu JR, Antos CL, et al. A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. Cell 1998;93:215–28.

8. Bueno OF, Wilkins BJ, Tymitz KM, et al. Impaired cardiac hypertrophic response in Calcineurin Abeta-deficient mice. Proc Natl Acad Sci U S A 2002;99:4586–91.

9. Lara-Pezzi E, Menasché P, Trouvin J-H, et al. Guidelines for translational research in heart failure. J Cardiovasc Transl Res 2015;8:3–22.

10. Ben-Sahra I, Hoxhaj G, Ricoult SJH, Asara JM, Manning BD. mTORC1 induces purine synthesis through control of the mitochondrial tetrahydrofolate cycle. Science 2016;351:728–33.

11. DeNicola GM, Chen P-H, Mullarky E, et al. NRF2 regulates serine biosynthesis in non-small cell lung cancer. Nat Genet 2015;47:1475–81.

12. Yang M, Vousden KH. Serine and one-carbon metabolism in cancer. Nat Rev Cancer 2016;16:650–62.

13. Martinez-Acedo P, Nunez E, Gomez FJ, et al. A novel strategy for global analysis of the dynamic thiol redox proteome. Mol Cell Proteomics 2012;11:800–13.

14. Pacold ME, Brimacombe KR, Chan SH, et al. A PHGDH inhibitor reveals coordination of serine synthesis and one-carbon unit fate. Nat Chem Biol 2016;12:452–8.

15. Zhang Y, Sano M, Shinmura K, et al. 4-Hydroxy-2-nonenal protects against cardiac ischemia–reperfusion injury via the Nrf2-dependent pathway. J Mol Cell Cardiol 2010;49:576–86.

16. Braz JC, Bueno OF, Liang Q, et al. Targeted inhibition of p38 MAPK promotes hypertrophic cardiomyopathy through upregulation of calcineurin-NFAT signaling. J Clin Invest 2003;111:1475–86.

17. Liu W, Zi M, Jin J, et al. Cardiac-specific deletion of Mkk4 reveals its role in pathological hypertrophic remodeling but not in physiological cardiac growth. Circ Res 2009;104:905–14.

18. Liang Q, Bueno OF, Wilkins BJ, Kuan CY, Xia Y, Molkentin JD. c-Jun N-terminal kinases (JNK) antagonize cardiac growth through cross-talk with calcineurin-NFAT signaling. EMBO J 2003;22:5079–89.

19. Ducker GS, Rabinowitz JD. One-carbon metabolism in health and disease. Cell Metab 2017;25:27–42.

20. Possemato R, Marks KM, Shaul YD, et al. Functional genomics reveal that the serine synthesis pathway is essential in breast cancer. Nature 2011;476:346–50.

21. Bao XR, Ong S-E, Goldberger O, et al. Mitochondrial dysfunction remodels one-carbon metabolism in human cells. eLife 2016;5:e10575.

22. Nikkanen J, Forsström S, Euro L, et al. Mitochondrial DNA replication defects disturb cellular dNTP pools and remodel one-carbon metabolism. Cell Metab 2016;23:635–48.

23. Watanabe Y, Watanabe K, Kobayashi T, et al. Chronic depletion of glutathione exacerbates ventricular remodelling and dysfunction in the pressure-overloaded heart. Cardiovasc Res 2013;97:282–92.

KEY WORDS cardiac function, cell signaling, hypertrophy, metabolism

APPENDIX For an expanded Methods section as well as supplemental figures and tables, please see the online version of this article.