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Elevated MCU expression by CaMKII δ B limits pathological cardiac remodeling

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Abstract

Background: Calcium (Ca²⁺) is a key regulator of energy metabolism. Impaired Ca²⁺ homeostasis damages mitochondria, causing cardiomyocyte death, pathological hypertrophy, and heart failure. This study investigates the regulation and the role of the mitochondrial Ca²⁺ uniporter (MCU) in chronic stress-induced pathological cardiac remodeling.

Methods: MCU knockout or transgenic mice were infused with isoproterenol (ISO, 10 mg/kg/day, 4 weeks). Cardiac hypertrophy and remodeling were evaluated by echocardiography and histology. Primary cultured rodent adult cardiomyocytes were treated with ISO (1 nM, 48 hr). Intracellular Ca²⁺ handling and cell death pathways were monitored. Adenovirus-mediated gene manipulations were used *in vitro*.

Results: Chronic administration of the β -adrenergic receptor (β -AR) agonist ISO increased the levels of the MCU and the MCU complex in cardiac mitochondria, raising mitochondrial Ca²⁺ concentrations, *in vivo* and *in vitro*. ISO also upregulated MCU without affecting its regulatory proteins in adult cardiomyocytes. Interestingly, ISO-induced cardiac hypertrophy, fibrosis, contractile dysfunction, and cardiomyocyte death were exacerbated in global MCU knockout (KO) mice. Cardiomyocytes from KO mice or mice overexpressing a dominant negative MCU exhibited defective intracellular Ca²⁺ handling and activation of multiple cell death pathways. Conversely, cardiac-specific overexpression of MCU maintained intracellular Ca²⁺ homeostasis and contractility, suppressed cell death, and prevented ISO-induced heart hypertrophy. ISO

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Disclosures
None.

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upregulated MCU expression through activation of Ca²⁺/calmodulin kinase II δ B (CaMKII δ B) and promotion of its nuclear translocation via calcineurin-mediated dephosphorylation at serine 332. Nuclear CaMKII δ B phosphorylated cAMP-response element binding protein (CREB), which bound the MCU promoter to enhance MCU gene transcription.

Conclusions: The β -AR/CaMKII δ B/CREB pathway upregulates MCU gene expression in the heart. MCU upregulation is a compensatory mechanism that counteracts stress-induced pathological cardiac remodeling by preserving Ca²⁺ homeostasis and cardiomyocyte viability.

Keywords

Animal Models of Human Disease; Basic Science Research; Cell Biology/Structural Biology; Metabolism; Heart Failure; Mitochondrial Ca²⁺ uniporter; Calcium calmodulin kinase II δ B; β -adrenergic receptor; Cardiomyocyte death; Pathological cardiac hypertrophy

Introduction

Impaired mitochondrial energy metabolism is a key contributor to human disease, including heart failure.¹ Mitochondrial Ca²⁺ has a critical role in regulating energy metabolism, and dysregulated Ca²⁺ can cause mitochondrial damage and even cell death.^{2,3} The mitochondrial Ca²⁺ uniporter (MCU), which forms a complex with its regulatory proteins, is responsible for mitochondrial Ca²⁺ uptake across the inner membrane.⁴⁻⁶ Although, MCU may be dispensable for heart function and energetics under normal conditions, it participates in metabolic adaptation during acute β -adrenergic receptor (β -AR) activation and ischemia-reperfusion injury in the heart.⁷⁻⁹ Until now, the regulation and role of MCU in chronic stress-induced pathological cardiac remodeling had remained poorly understood.⁶ MCU is upregulated in the heart by exercise and pressure overload, suggesting that its function is complicated and depends on the context.¹⁰ In addition, either excessive or absent MCU-mediated Ca²⁺ uptake can cause cell death and mitochondrial damage.^{11,12} For instance, MCU knockout (KO) increases Ca²⁺ sensitivity of the mitochondrial permeability transition pore (mPTP).¹¹ However, transcriptional, translational, and post-translational regulation of MCU under chronic pathological conditions have not been thoroughly investigated.^{10,12-14}

β -adrenergic receptor (β -AR) signaling is the most powerful regulatory mechanism in the heart, as it underlies both the acute fight-or-flight response and chronic stress-induced cardiac pathology.¹⁵ β 1-AR is the major β -AR subtype that stimulates multiple signaling pathways, including cyclic adenosine monophosphate / protein kinase A (cAMP/PKA), exchange protein directly activated by cAMP, calcineurin, and Ca²⁺/calmodulin kinase II (CaMKII).^{16,17} Chronic β 1-AR activation leads to heart hypertrophy and dysfunction mainly through CaMKII.¹⁸ One major CaMKII subtype is CaMKII δ C, which induces cardiomyocyte death and mitochondrial dysfunction.¹⁹ However, CaMKII δ B, another major CaMKII subtype, may benefit the heart through mechanisms that are not fully established.²⁰

Here, we demonstrated that the β -AR/CaMKII δ B/cAMP-response element binding protein (CREB) pathway increases MCU gene transcription in the stressed heart. By using gain- or loss-of-function approaches, we found that MCU upregulation maintains intracellular Ca²⁺ and energy homeostasis, prevents cell death, limits cardiac hypertrophy, and preserves heart

function during chronic stress. Therefore, MCU is a stress-responsive gene underlying the beneficial effects of CaMKII δ B in pathological cardiac remodeling.

Methods

Detailed methods are in the Online Supplemental Materials. The authors declare that all supporting data are available within the article and the Online Data Supplement. The data, analytic methods, and study materials will be made available to other researchers for purposes of reproducing the results or replicating the procedure. Raw data are available from the corresponding author on reasonable request.

Animals

All animal procedures were performed in accordance with the approved protocol by the Institutional Animal Care and Use Committee (IACUC) of the University of Washington. The experiments included in this study used both male and female mice.

Statistical Analysis

Data are shown as mean \pm standard error (SEM). When multiple experiments using different numbers of animals were pooled for statistical analysis, the range of animal numbers was indicated in the figure legend. Statistical analyses used GraphPad Prism 7.02 software. Shapiro-Wilk normality test was performed to determine the data distribution. Non-normally distributed data were analyzed by nonparametric tests: Kruskal-Wallis test followed by Dunn's post hoc analysis for >2 groups or Mann-Whitney test for 2 groups. Normally distributed data were analyzed by using parametric tests: one-way analysis of variance (ANOVA) followed by Tukey test for >2 groups or unpaired Student's t-test for 2 groups. Multiple testing adjustment was included in the Dunn's test and Tukey test through controlling the Type I error for the family of comparisons. Experiments consisting of two categorically independent variables and one dependent variable were analyzed by two-way ANOVA. A P value <0.05 was considered statistically significant.

Results

Chronic β -AR activation upregulated MCU and the MCU complex in cardiac mitochondria

The β -AR agonist isoproterenol (ISO, 10 mg/kg/day for 1–4 weeks) increased protein levels of MCU and MCU complex in the mitochondrial fraction and tissue homogenates of mouse heart (Figure 1A and Supplemental Figure 1A). Meanwhile, MCU regulatory proteins were unchanged, except for MCUB and EMRE, which decreased during weeks 1 and 4, respectively (Supplemental Figure 1A). In cultured mouse cardiomyocytes, ISO dose- and time-dependently elevated the level of MCU protein but had mild or no effects on MCU regulatory proteins (Figure 1B–C, Supplemental Figure 1B–C). ISO also upregulated MCU in adult rat cardiomyocytes and H9C2 cardiomyoblasts (Supplemental Figure 1D–E). Functionally, upregulation of MCU and MCU complex was accompanied by higher Ca²⁺ concentrations in cardiac mitochondria, both *in vivo* and *in vitro* (Figure 1D–E), and accelerated Ca²⁺ uptake by isolated mitochondria after each bolus Ca²⁺ addition (Figure

1F). These results suggest that chronic β -AR activation specifically targets MCU and promotes mitochondrial Ca^{2+} uptake in cardiomyocytes.

MCU KO exacerbated ISO-induced pathological cardiac hypertrophy

To explore the consequences of MCU upregulation in the heart, we used global MCU KO mice.⁷ MCU and MCU complex were absent from KO mitochondria; however, MCU regulatory proteins were not significantly changed (Figure 2A and Supplemental Figure 2A). MCU KO mitochondria did not take up Ca^{2+} and had lower levels of free Ca^{2+} in the matrix (Supplemental Figure 2B–C). Unexpectedly, ISO (10 mg/kg/day, 4 weeks)-induced heart hypertrophy (heart weight to body weight ratio, heart weight to tibia length ratio, atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) mRNA levels, and cross-sectional area of cardiomyocytes), contractile dysfunction (ejection fraction and fractional shortening), and fibrosis (Picrosirius red staining and *Col1a2* mRNA level) were all exacerbated in KO heart (Figure 2B–H and Supplemental Figure 2D–G). In addition, ISO induced apoptosis (caspase 3/7 activity) in KO heart tissue and higher levels of cell death in cultured adult cardiomyocytes from KO mice (Figure 2I and Supplemental Figure 2H). These results indicate that the lack of MCU potentiates, rather than ameliorates, pathological cardiac remodeling induced by chronic β -AR activation.

To determine whether the above phenotypes in global MCU KO hearts are cardiomyocyte-specific, we crossed MCU^{fl/fl} mice with α MHC-MerCreMer (MCM) mice to generate cardiomyocyte-specific MCU KO mice (cKO, Supplemental Figure 3A).^{8,9} ISO (10 mg/kg/day, 4 weeks)-induced heart hypertrophy, contractile dysfunction, fibrosis, and apoptosis were also exacerbated in cKO hearts (Supplemental Figure 3B–I), suggesting that the lack of MCU in cardiomyocytes facilitates pathological cardiac remodeling.

MCU KO impaired intracellular Ca^{2+} handling and activated cell death pathways

To investigate why MCU KO exacerbated pathological cardiac remodeling, we first found that ISO increased the sensitivity of laser-induced mitochondrial permeability transition pore (mPTP) openings similarly in cultured wild-type (WT) and KO cardiomyocytes (Figure 2J), suggesting that MCU KO does not further activate the mitochondrial cell death pathway. Next, we explored Ca^{2+} handling in cardiomyocytes after the 4-week period of ISO administration. As expected, ISO elevated mitochondrial Ca^{2+} concentrations (measured by mt-PeriCam) in WT, but not in KO, cardiomyocytes (Figure 2K and Supplemental Figure 4A). Interestingly, ISO increased diastolic cytosolic Ca^{2+} concentrations (measured by Twitch2B), decreased sarcoplasmic reticulum (SR) Ca^{2+} content (measured by D1ER), and suppressed the SR Ca^{2+} uptake rate (decay of caffeine-induced Ca^{2+} transients) significantly more in MCU KO cardiomyocytes compared to WT (Figure 2L–N and Supplemental Figure 4B–D), indicating that MCU KO magnified intracellular Ca^{2+} dysregulation. Because SR stress markers and sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) 2 levels were comparable in WT and KO hearts (Supplemental Figure 4E–F), the decreased SR Ca^{2+} uptake rate in MCU KO cardiomyocytes might be due to compromised SERCA function.

Subsequently, we determined whether the elevated cytosolic Ca^{2+} concentrations were responsible for increased cell death in KO mouse heart tissue. Chelation of cytosolic Ca^{2+}

with BAPTA-AM (0.1 $\mu\text{mol/L}$) suppressed ISO-induced caspase 3/7 activation in both WT and MCU KO cardiomyocytes (Supplemental Figure 4G and Figure 2O). However, ISO increased the activity of calpain,^{21,22} a protease activated by high levels of Ca^{2+} , only in KO cardiomyocytes, and BAPTA treatment abolished calpain activation (Supplemental Figure 4H and Figure 2P). Moreover, calpain inhibitor XI (Cal XI) blocked ISO-induced activation of calpain and caspase 3/7 in KO cardiomyocytes (Supplemental Figure 4I–J and Figure 2Q). Finally, increased calpain activity was found in KO or cKO, but not control, hearts after the 4-week period of ISO administration (Figure 2R and Supplemental Figure 4K). These results suggest that cytosolic Ca^{2+} overload and calpain activation may mediate the increased cell death observed in MCU KO cardiomyocytes.

MCU maintained Ca^{2+} and energy homeostasis in cardiomyocytes

To explore how MCU-mediated mitochondrial Ca^{2+} uptake regulates intracellular Ca^{2+} homeostasis and cell viability, we inhibited or enhanced MCU function in cultured adult rat cardiomyocytes. First, overexpressing a dominant negative MCU (DNMCU, Supplemental Figure 5A)⁵ recapitulated the dysregulated intracellular Ca^{2+} handling found in MCU KO cardiomyocytes. DNMCU attenuated mitochondrial Ca^{2+} uptake in permeabilized cardiomyocytes, reduced matrix Ca^{2+} levels at baseline, and abolished ISO (0.1 $\mu\text{mol/L}$, 24 hr)-induced mitochondrial Ca^{2+} elevation (Supplemental Figure 5B and Figure 3A). In cultured rat cardiomyocytes, ISO (0.1 $\mu\text{mol/L}$, 24 hr) had no effect on cytosolic or SR Ca^{2+} content but decreased the amplitude and decay velocity of pacing-induced Ca^{2+} transients (Figure 3B–D and Supplemental Figure 5C–D). In DNMCU-overexpressing cells, ISO elevated cytosolic Ca^{2+} concentrations, lowered SR Ca^{2+} content, and further decreased the amplitude and decay velocity of pacing-induced Ca^{2+} transients (Figure 3B–D and Supplemental Figure 5C–D). Conversely, overexpression of FLAG-tagged MCU in adult rat cardiomyocytes elevated mitochondrial Ca^{2+} concentrations, lowered cytosolic Ca^{2+} concentrations, and increased SR Ca^{2+} content at baseline and after ISO incubation (Figure 3E–H).

DNMCU overexpression activated calpain and further enhanced caspase 3/7 activity; however, MCU overexpression exhibited no calpain activation and attenuated caspase 3 activation after ISO incubation (Figure 3I–N). Finally, we used an ATP indicator (ATeam) to monitor steady-state ATP levels in cultured rat cardiomyocytes.²³ ISO elevated cytosolic ATP levels in control, but not in DNMCU-overexpressing, cardiomyocytes (Figure 3O). Moreover, the free mitochondrial ATP level (mt-ATeam) was increased by ISO, while DNMCU abolished this effect (Supplemental Figure 5E and Figure 3P). Mitochondrial respiration trended higher after ISO incubation or MCU overexpression, and lower after DNMCU overexpression in adult rat cardiomyocytes (Supplemental Figure 5F). Taken together, our results suggest that inhibition of MCU impairs, whereas overexpression of MCU benefits, energy and Ca^{2+} homeostasis in cardiomyocytes during chronic stress.

MCU transgenic mice were protected during chronic β -AR activation

To determine the significance of MCU upregulation in stress-induced heart dysfunction *in vivo*, we generated inducible, heart-specific MCU transgenic (TG) mice, which express FLAG-tagged MCU (MCU-Flag) after tamoxifen (5 mg/kg/day for 2 days) induction

(Supplemental Figure 6A–B). MCU TG mice had increased levels of MCU complex and decreased phosphorylation of pyruvate dehydrogenase in cardiac mitochondria, but they had no differences in MCU regulatory proteins, mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCLX), and SERCA2a levels (Figure 4A and Supplemental Figure 6C–D), which is consistent with a central role of MCU in MCU complex assembly and mitochondrial energy metabolism. Notably, mitochondrial Ca^{2+} uptake assays revealed that isolated mitochondria from MCU TG heart tissue took up more Ca^{2+} after each bolus Ca^{2+} addition and at a faster rate than WT mitochondria (Figure 4B).

ISO (10 mg/kg/day, 4 weeks)-induced cardiac hypertrophy, reduced contractility, and fibrosis were all attenuated in MCU TG mouse hearts compared to the control floxed/floxed Stop polyA MCU (F/F) or α MHC-MCM (MCM) mouse hearts (Figure 4C–G, Supplemental Figure 7A–E, and Supplemental Figure 8A–H). At baseline, adult cardiomyocytes from TG mice exhibited higher mitochondrial Ca^{2+} and lower cytosolic Ca^{2+} concentrations, as well as unchanged SR Ca^{2+} content, pacing-induced Ca^{2+} transients, and SR Ca^{2+} uptake rate (Figure 4H–L). After the 4-week period of ISO administration, mitochondrial Ca^{2+} concentration remained high and cytosolic Ca^{2+} concentration remained low in TG cardiomyocytes (Figure 4H–I). The SR Ca^{2+} content, pacing-induced Ca^{2+} transients, and SR Ca^{2+} uptake rate were unchanged in TG but decreased in F/F cardiomyocytes after ISO administration (Figure 4J–L). These results are consistent with the observations from MCU-overexpressing cardiomyocytes, indicating that upregulating MCU maintains intracellular Ca^{2+} homeostasis, prevents cytosolic Ca^{2+} overload, and facilitates SR Ca^{2+} uptake. Finally, the total ATP level was unchanged, calpain was not activated, and caspase 3/7 activity was suppressed in TG compared to F/F or MCM hearts after the 4-week ISO administration (Figure 4M–N, Supplemental Figure 7F, and Supplemental Figure 8I–J). In addition, whereas mPTP opening was suppressed in the TG heart, NCLX and SR stress markers were comparable in TG and F/F heart after ISO administration (Supplemental Figure 7G–I). In summary, overexpression of MCU protects the heart from stress-induced pathological hypertrophy through enhancing mitochondrial Ca^{2+} uptake and cytosolic Ca^{2+} cycling, which suppresses cell death pathways.

CaMKII δ B and calcineurin upregulated MCU

Next, we explored the signaling pathway responsible for MCU upregulation. First, we screened downstream signaling pathways of β -AR. Overexpressing PKA inhibitory peptide (PKI) did not affect, whereas CaMKII inhibitor KN93 (0.5 $\mu\text{mol/L}$) abolished, ISO-induced MCU upregulation in adult mouse cardiomyocytes (Figure 5A–B and Supplemental Figure 9A). In order to bypass the receptor, a constitutively active $\text{G}\alpha$ (caG α),²⁴ which stimulates downstream signaling pathways of β -AR, was over expressed. caG α activated CaMKII through autophosphorylation of threonine 287 (T287), resulting in upregulation of MCU in a KN93-dependent manner (Figure 5C and Supplemental Figure 9B). Furthermore, shRNA-mediated CaMKII knockdown (shCaMKII) inhibited ISO-induced MCU upregulation (Figure 5D and Supplemental Figure 9C). These results suggest that CaMKII, but not PKA, is responsible for ISO-induced MCU upregulation.

Next, we determined which CaMKII subtype upregulates MCU. Adenovirus-mediated overexpression of HA-tagged CaMKII δ B, but not CaMKII δ C, increased MCU protein and mRNA levels in adult cardiomyocytes (Figure 5E and Supplemental Figure 9D–E). Conversely, HA-tagged dominant negative CaMKII δ B (DN δ B), but not DN δ C, blocked ISO-induced MCU upregulation (Figure 5F and Supplemental Figure 9F). Meanwhile, DN δ B also abolished the elevated mitochondrial Ca²⁺ concentrations induced by ISO (Figure 5G). Finally, we overexpressed δ B or δ C in adult cardiomyocytes isolated from CaMKII δ KO mice²⁵ and found that δ B, but not δ C, elevated MCU expression (Supplemental Figure 10). Taken together, our results suggest that CaMKII δ B, but not δ C, is necessary and sufficient for ISO-induced MCU upregulation.

Calcineurin, also known as protein phosphatase 2B, was activated by ISO (Supplemental Figure 11A). Calcineurin inhibitor cyclosporine A (CsA, 1 μ mol/L) blocked ISO-induced MCU upregulation, but overexpressing WT calcineurin did not affect MCU protein levels in adult cardiomyocytes (Figure 5H–I and Supplemental Figure 11B–C), indicating that calcineurin is necessary but not sufficient for MCU upregulation. In addition, CsA or WT calcineurin did not affect CaMKII phosphorylation at T287 (Figure 5H–I and Supplemental Figure 11B–C). Lastly, β blocker propranolol (Prop, 10 mg/kg/day) or CaMKII inhibitor KN93 (10 μ mol/kg/day) abolished ISO-induced MCU upregulation in mouse heart tissue *in vivo* (Figure 5J).

Nuclear translocation of activated CaMKII δ B upregulated MCU expression

CaMKII δ B and δ C are alternative splicing products of the CaMKII δ gene. They share an identical sequence, except that δ B has an 11-amino acid nuclear localization sequence (NLS).²⁶ Because δ B is distributed in different compartments of cardiomyocytes,²⁷ we first determined whether nuclear δ B regulates MCU expression. Targeting a green fluorescent protein (GFP)-tagged autocalmitide-2-related inhibitory peptide (AIP, a CaMKII inhibitor) to the cytosol (c-GFP-AIP) or nucleus (n-GFP-AIP) abolished ISO-induced CaMKII phosphorylation at T287 in the corresponding compartments (Supplemental Figure 12A). However, only n-GFP-AIP, but not c-AIP, blocked ISO-induced MCU upregulation in adult mouse cardiomyocytes (Supplemental Figure 12B). Similarly, targeting a red fluorescent protein (RFP)-tagged CaMKII inhibitory protein to the cytosol (c-CaMKIIN) or nucleus (n-CaMKIIN) attenuated ISO-induced CaMKII activation (T287 phosphorylation) in the corresponding compartments (Figure 6A and Supplemental Figure 12C), but only n-CaMKIIN prevented ISO-induced MCU upregulation (Figure 6B). Taken together, our results suggest that nuclear CaMKII δ B regulates MCU gene expression.

Because the phosphorylation status of serine 332 (S332), which is located near the NLS, controls intracellular localization of CaMKII δ B,²⁸ we tested whether S332 dephosphorylation promotes its nuclear translocation and regulation of MCU expression. First, ISO-induced nuclear translocation of HA-tagged CaMKII δ B was blocked by CsA (Figure 6C). Conversely, overexpressing calcineurin promoted nuclear translocation of CaMKII δ B (Figure 6D and Supplemental Figure 12D). Second, we overexpressed HA-tagged and phosphorylation mimetic (S332E) or unphosphorylated (S332A) mutated CaMKII δ B in adult cardiomyocytes. S332E stayed in the cytosol with or without ISO

incubation, whereas S332A was located inside the nucleus even in the presence of CsA (Supplemental Figure 12E). Importantly, S332E abolished ISO-induced MCU upregulation, whereas S332A upregulated MCU despite the presence of CsA (Supplemental Figure 12F). These results support our hypothesis that calcineurin dephosphorylates CaMKII δ B at S332 to facilitate its nuclear translocation for regulation of MCU expression.

Next, we determined whether calcineurin directly dephosphorylates CaMKII δ B at S332. First, S332 phosphorylation was abundantly detected by mass spectrometry in purified CaMKII δ B but not after calcineurin incubation (Figure 6E). In addition, calcineurin bound CaMKII δ B (Figure 6F) and dephosphorylated it at S332 (detected by using an antibody against phosphorylated S332) in a cell-free system and in adult cardiomyocytes after ISO incubation or calcineurin overexpression (Supplemental Figure 13A–B). Furthermore, CsA blocked ISO- or calcineurin-induced S332 dephosphorylation (Supplemental Figure 13C). CaMKII δ B phosphorylation at S332 was detected only in the cytosol (Supplemental Figure 13D). Overexpressing a dominant negative calmodulin attenuated ISO-induced MCU upregulation (Supplemental Figure 13E), consistent with the role of calmodulin in activating both CaMKII and calcineurin. In whole-heart samples from mice after 4 weeks of ISO administration or from heart failure patients, we confirmed the decrease in CaMKII δ B S332 phosphorylation, increase in CaMKII δ B T287 phosphorylation, and elevated MCU protein levels (Figure 6G–H and Supplemental Figure 13F–G). Taken together, these results suggest that calcineurin dephosphorylates CaMKII δ B at S332 to facilitate its nuclear translocation and MCU upregulation.

CaMKII δ B phosphorylated CREB to promote MCU transcription

ISO increased MCU mRNA levels in a KN93-dependent manner and enhanced the activity of the MCU promoter (Supplemental Figure 14A–B). Bioinformatics analysis revealed four sequences in the MCU promoter region that are highly homologous to the consensus cAMP response element (CRE I, II, III, and IV) (Supplemental Figure 14C–D). A Luciferase reporter system containing a truncated MCU promoter or CRE mutants showed that CRE IV was responsible for ISO-induced MCU gene transcription (Figure 7A–B). Next, chromatin immunoprecipitation assays and electrophoretic mobility shift assays, respectively, revealed that CREB bound CRE IV in adult cardiomyocytes and *in vitro* (Figure 7C–E). Moreover, ISO increased CREB phosphorylation at its activating site (S133) in a KN93-dependent manner (Figure 7F and Supplemental Figure 14E). Co-immunoprecipitation detected that endogenous CREB and CaMKII bound each other in adult cardiomyocytes, and purified CaMKII δ B bound and phosphorylated CREB at S133 *in vitro* (Figure 7G–H and Supplemental Figure 14F–G). A dominant negative mutation (K43A, in the ATP binding domain) of CaMKII δ B, but not S332E or S332A, failed to phosphorylate CREB (Figure 7I), suggesting that the phosphorylation status of S332 does not affect the kinase activity of CaMKII δ B and the kinase activity is needed for CREB phosphorylation. Finally, overexpression of a dominant negative CREB (S133A) abolished ISO or CaMKII δ B's effects on MCU mRNA and protein levels and on mitochondrial Ca²⁺ concentrations in adult cardiomyocytes (Figure 7J–L and Supplemental Figure 14H), suggesting that CREB phosphorylation mediates ISO/CaMKII δ B-induced MCU expression.

These results indicate that CaMKII δ B phosphorylates CREB at S133 to modulate MCU gene transcription.

Discussion

In this study, we discovered a novel mechanism, which is centered on the MCU, to limit pathological cardiac remodeling (Figure 8). During chronic β -AR stimulation, the CaMKII δ B/CREB pathway upregulates MCU gene transcription, leading to increased formation of the MCU complex and mitochondrial Ca²⁺ uptake. MCU-mediated Ca²⁺ uptake orchestrates Ca²⁺ handling among mitochondria, SR, and cytosol to ameliorate cytosolic Ca²⁺ overload-induced cardiomyocyte death. MCU KO worsens, whereas MCU transgenic overexpression prevents, heart hypertrophy and dysfunction. Therefore, enhancing MCU-mediated mitochondrial Ca²⁺ uptake could be a new approach to treat heart failure.

Pathophysiological functions of MCU, the major mitochondrial Ca²⁺ uptake channel,^{4,5} in the heart remain elusive, because genetic manipulations of MCU have yielded conflicting results.⁶ For instance, mitochondrial Ca²⁺ overload is known to mediate acute ischemia-reperfusion injury in the heart through boosting respiration, reactive oxygen species production, and mPTP opening.²⁹ However, reperfusion injury of the heart is not ameliorated in all MCU KO models.⁷⁻⁹ Chronic stress-induced heart dysfunction is linked to either mitochondrial Ca²⁺ overload or reduced Ca²⁺ uptake.^{3,30} Consistent with this notion, a recent report showed that increasing MCU levels rescues heart failure in guinea pigs.³¹ However, MCU KO does not prevent pressure overload-induced heart hypertrophy and failure in mice,³² casting doubts on the role of endogenous MCU in the failing heart. Here, we showed that MCU KO or TG oppositely impacted ISO-induced heart remodeling, providing explicit evidence to support a critical role of MCU in the chronically stressed heart. Future studies are needed to elucidate whether MCU plays similar or distinct roles in different heart failure models and/or at different disease stages.

Our results highlight that regulation of cytosolic Ca²⁺ is a new mechanism by which mitochondrial Ca²⁺ participates in heart failure development. Unlike in other cell types, such as skeletal muscle and some cell lines,^{33,34} mitochondria in cardiomyocytes play a negligible role in directly buffering cytosolic Ca²⁺ under physiological conditions.⁷ We found that during chronic adrenergic stress, increased mitochondrial Ca²⁺ uptake limits cytosolic Ca²⁺ overload indirectly through boosting energy metabolism and SR Ca²⁺ reuptake. Importantly, when mitochondrial Ca²⁺ uptake is further and persistently enhanced, as in the MCU TG mice, cytosolic Ca²⁺ overload and pathological heart hypertrophy are completely prevented. Interestingly, we detected increased ATP levels in cultured cells but not in heart tissue when mitochondrial Ca²⁺ uptake is enhanced. This is likely because that increased energy production is fully and promptly matched by increased workload in the beating heart but not in the quiescent cells. Mitochondrial respiration was not compromised by ISO incubation, suggesting the lack of significant mitochondrial dysfunction, which could be induced by more robust adrenergic stress³⁵.

Our study also revealed a central role of MCU in the assembly of the MCU complex in cardiomyocytes. The MCU complex is the functional unit for mitochondrial Ca^{2+} uptake.⁵ A noteworthy finding is that the levels of MCU and MCU complex, as well as the mitochondrial Ca^{2+} uptake rate, are modulated concomitantly by ISO stimulation and in MCU TG or KO hearts. In contrast, the levels of MCU regulatory proteins are mostly unchanged. Therefore, MCU and its regulatory proteins are independently regulated by ISO stimulation in the heart and manipulating MCU is sufficient to influence the formation of functional MCU complex. Thus, MCU could be a stress response gene whose upregulation quickly leads to enhanced mitochondrial Ca^{2+} uptake. It should be noted that ISO induces different changes in some of the MCU regulatory proteins in cultured cells and the heart. Future studies are needed to explore whether this is due to the different dosages and/or duration of ISO stimulation or the different conditions of cardiomyocytes (*in vitro* versus *in vivo*).

Finally, this study delineated a molecular mechanism by which CaMKII δ B controls MCU gene expression in the heart. Chronic β -AR stimulation activates both the δ B and δ C subtypes of CaMKII, which have unique functions in the heart.³⁶ In particular, CaMKII δ B regulates the expression of genes associated with hypertrophic cardiomyopathy and leads to compensated hypertrophy, whereas CaMKII δ C causes pathological hypertrophy with impaired heart function.^{20,37,38} The exact mechanisms or targets of CaMKII δ B responsible for maintaining cardiac function in the hypertrophic heart are unknown. Previous reports showed that CaMKII regulates gene expression through CREB in neurons and that CREB modulates MCU gene expression in cancer cells.^{14,39} Our results suggest that CaMKII δ B regulates MCU gene transcription through phosphorylating CREB at S133 in cardiomyocytes. Consistent with our findings, mice expressing dominant-negative CREB (S133A) in the heart exhibited dilated cardiomyopathy with mitochondrial dysfunction.^{40,41} Moreover, we found that both nuclear translocation through S332 dephosphorylation and kinase activation through T287 phosphorylation are needed for CaMKII δ B to upregulate MCU gene expression (Figure 8). Previous reports showed that CaMKI or CaMKIV phosphorylates CaMKII at S332, keeping it in the cytosol, whereas phosphatases (PP1 α and PP1 γ 1) dephosphorylate CaMKII and promote its nuclear translocation.^{28,42} We found that calcineurin can also dephosphorylate CaMKII δ B at S332 to facilitate its nuclear translocation. Importantly, the dual mechanisms occur independently and act synergistically. Because both calcineurin and CaMKII are Ca^{2+} - and calmodulin-dependent, elevated cytosolic Ca^{2+} concentrations might be the initial trigger of the CaMKII δ B/CREB/MCU pathway, which serves as a negative feedback mechanism to limit cytosolic Ca^{2+} overload, maintain cell viability, and prevent pathological remodeling.

In summary, the MCU is transcriptionally upregulated by the β -AR/CaMKII δ B/CREB pathway to maintain intracellular Ca^{2+} and energy homeostasis as well as cell viability (Figure 8). Targeting the CaMKII δ B/CREB/MCU axis may be a novel approach to counteract chronic stress-induced pathological cardiac remodeling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Non-standard Abbreviations and Acronyms

ANP	atrial natriuretic peptide
β-AR	β-adrenergic receptor
BNP	brain natriuretic peptide
BW	body weigh
caGsa	constitutive active G protein sa
Cal XI	calpain inhibitor XI
CaM	Calmodulin
CaMKII	Ca ²⁺ calmodulin kinase II
cAMP	cyclic adenine monophosphate
CaN	Calcineurin
ChIP	chromatin immunoprecipitation
cKO	cardiomyocyte-specific MCU knockout mice
Col1a2	collagen type I α 2 chain
CRE	cAMP response element
CREB	cAMP-response element binding protein
CsA	cyclosporine A
DNCaM	dominant negative calmodulin
DNCREB	dominant negative CREB
DNMCU	dominant negative MCU
EMRE	essential mitochondrial calcium uniporter regulator
EMSA	electrophoretic mobility shift assay

F/F	MCU floxed/floxed
GFP	green fluorescent protein
HW	heart weight
ISO	Isoproterenol
KHB	Krebs-Henseleit Buffer
KO	global MCU knockout mice
MCM	α .MHC-MerCreMer mice
MCU	mitochondrial calcium uniporter
MCUb	mitochondrial calcium uniporter regulatory subunit b
MICU1	mitochondrial calcium uptake 1
mPTP	mitochondrial permeability transition pore
MS	mass spectrometry
NLS	nuclear localization sequence
PKA	protein kinase A
PKI	PKA inhibitory peptide
RFP	red fluorescent protein
SERCA2a	sarco/endo plasmic reticulum Ca^{2+} ATPase 2a
SR	sarcoplasmic reticulum
TG	heart specific and inducible MCU transgenic mice
TL	tibia length
WT	wild type mice

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Clinical Perspective

What is new?

- MCU is upregulated in the stressed heart to orchestrate mitochondrial, sarcoplasmic reticulum, and cytosolic Ca^{2+} handling, preventing cytosolic Ca^{2+} overload-induced cardiomyocyte death.
- Lack of MCU-mediated mitochondrial Ca^{2+} uptake is detrimental, whereas transgenic overexpression MCU is beneficial, to the heart during chronic β adrenergic stimulation.
- The nuclear translocation of CaMKII δ B, via calcineurine-mediated dephosphorylation of serine 332, activates CREB to promote MCU gene expression in adult cardiomyocytes.

What are the clinical implications?

- Our study indicates that enhancing mitochondrial Ca^{2+} uptake could be a new approach to prevent chronic β adrenergic stimulation-induced heart remodeling.
- Targeting the CaMKII δ B/CREB/MCU pathway could be a therapeutic opportunity for pathological cardiac remodeling associated with chronic adrenergic stress.

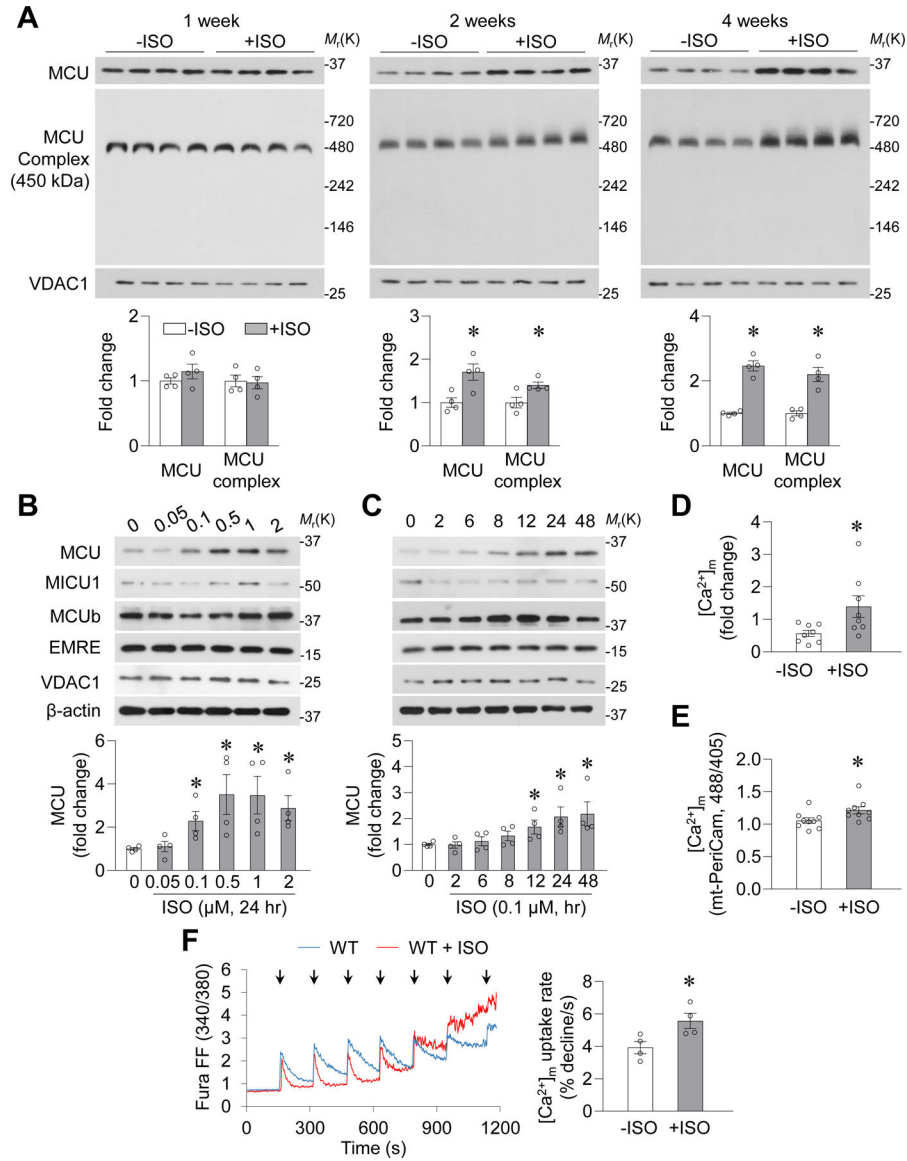


Figure 1. Chronic β -AR activation upregulated MCU in the heart and in cardiomyocytes. **A**, MCU and MCU complex measured in cardiac mitochondria after isoproterenol (+ISO, 10 mg/kg/day) or vehicle (-ISO) administration in mice for 1, 2 (*: $P=0.018$ or 0.0313 vs. -ISO for MCU or MCU complex, $n=4$), and 4 weeks (*: $P=8.6E-05$ or 0.0021 vs. -ISO for MCU or MCU complex, $n=4$). **B-C**, Dose- and time-dependent changes in MCU and associated proteins in adult mouse cardiomyocytes after ISO incubation. In **B**, *: $P=0.0294$, 0.0352 , 0.0296 , or 0.0176 vs. -ISO for 0.1, 0.5, 1 or 2 $\mu\text{mol/L}$ ISO groups, respectively. In **C**, *: $P=0.046$, 0.0309 or 0.0438 vs -ISO for 12, 24 or 48 hr ISO groups, respectively. $n=4$. **D**, Mitochondrial matrix free Ca^{2+} concentrations ($[\text{Ca}^{2+}]_m$) determined by rhod-2 and FCCP-induced mitochondrial Ca^{2+} release in freshly isolated and permeabilized adult mouse cardiomyocytes after 4-week ISO administration. *: $P=0.032$ vs. -ISO. $n=8$ mice. **E**, Steady state mitochondrial Ca^{2+} concentrations measured by mt-PeriCam in adult rat cardiomyocytes after ISO (0.1 $\mu\text{mol/L}$, 24 hr) incubation. *: $P=0.036$ vs. -ISO. $n=9$ rats.

F, Representative traces and summarized data showing Ca^{2+} uptake by freshly isolated mitochondria from the hearts of WT mice with or without *in vivo* ISO stimulation (ISO, 10 mg/kg/day, 4 weeks). Arrows: additions of Ca^{2+} (25 $\mu\text{mol/L}$ each time). *: $P=0.0337$ vs. -ISO, $n=4$ mice.

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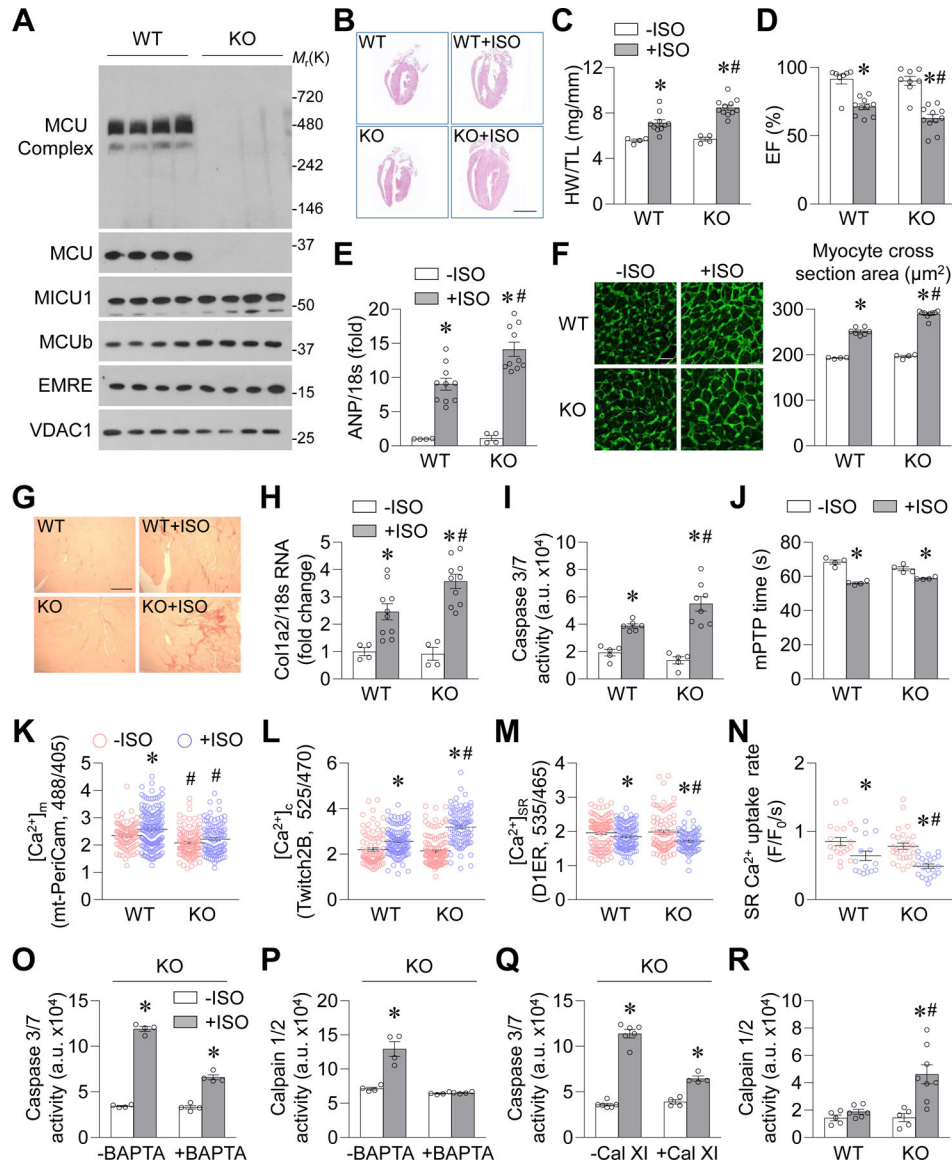


Figure 2. MCU KO exacerbated cardiac hypertrophy and dysfunction during chronic β -AR activation in mice.

A, Western blotting images showing MCU complex, MCU, and MCU regulatory proteins in mitochondrial fractions from wild type (WT) or MCU knockout (KO) mouse heart. *n*=4. **B**, Representative coronal sections of the heart with (+ISO) or without (-ISO) 4-week ISO administration (10 mg/kg/day). Scale bar = 5 mm. **C**, Heart weight to tibia length ratio (HW/TL). *: *P*=0.0053 WT+ISO vs. WT-ISO, #: *P*=9.57E-06 KO+ISO vs. KO-ISO, #: *P*=0.0013 KO+ISO vs. KO-ISO. *n*=5-6 for -ISO groups and *n*=10-11 for +ISO groups. **D**, Left ventricular ejection fraction. *: *P*=0.0001 WT+ISO vs. WT-ISO, #: *P*=5.84E-06 KO+ISO vs. KO-ISO, #: *P*=0.0246 KO+ISO vs. KO-ISO. *n*=7-11. **E**, mRNA levels of atrial natriuretic peptide (ANP) in mouse heart. *: *P*=0.0006 WT+ISO vs. WT-ISO, #: *P*=5.17E-05 KO+ISO vs. KO-ISO, #: *P*=0.0025 KO+ISO vs. WT+ISO. *n*=4 for -ISO groups and *n*=10 for +ISO groups. **F-G**, Wheat germ agglutinin staining for cardiomyocyte cross-sectional area (**F**, *: *P*=5.97E-07 WT+ISO vs. WT-ISO, #: *P*=5.73E-09 KO+ISO vs. WT+ISO).

KO-ISO, #: $P=1.17E-06$ KO+ISO vs. WT+ISO) and Picrosirius red staining for fibrosis (**G**). $n=4$ for -ISO groups and $n=7-9$ for +ISO groups. Scale bars = 100 μm . **H**, mRNA level of collagen type I $\alpha 2$ chain (Col1a2) in mouse heart. *: $P=0.0441$ WT+ISO vs. WT-ISO, *: $P=0.0004$ KO+ISO vs. KO-ISO, #: $P=0.0119$ KO+ISO vs. WT+ISO. $n=4$ for -ISO groups and $n=10$ for +ISO groups. **I**, Caspase 3/7 activity in mouse heart. *: $P=0.0003$ WT+ISO vs. WT-ISO, *: $P=0.0004$ KO+ISO vs. KO-ISO, #: $P=0.0322$ KO+ISO vs. WT+ISO. $n=5$ for -ISO groups and $n=6-8$ for +ISO groups. **J**, mPTP time in cardiomyocytes after ISO (0.1 $\mu\text{mol/L}$, 24 hr) incubation. *: $P=0.0003$ WT+ISO vs. WT-ISO, *: $P=0.0061$ KO+ISO vs. KO-ISO. $n=4$ mice. **K-N**, Mitochondrial Ca^{2+} concentrations (**K**, $[\text{Ca}^{2+}]_m$, measured by mt-PeriCam, *: $P=0.0048$ WT+ISO vs. WT-ISO, #: $P=7E-05$ KO-ISO vs. WT-ISO, #: $P=2.66E-05$ KO+ISO vs. WT+ISO, $n=97-157$ cells from 4-5 mice), cytosolic Ca^{2+} concentrations (**L**, $[\text{Ca}^{2+}]_c$, measured by Twitch2B, *: $P=9.37E-05$ WT+ISO vs. WT-ISO, *: $P=2.6E-22$ KO+ISO vs. KO-ISO, #: $P=6.5E-10$ KO+ISO vs. WT+ISO, $n=87-152$ cells from 4-5 mice), SR Ca^{2+} content (**M**, $[\text{Ca}^{2+}]_{\text{SR}}$, measured by D1ER, *: $P=0.0485$ WT+ISO vs. WT-ISO, *: $P=0.0002$ KO+ISO vs. KO-ISO, #: $P=0.0054$ KO+ISO vs. WT+ISO, $n=78-126$ cells from 4-5 mice), and SR Ca^{2+} uptake rate (**N**, *: $P=0.0291$ WT+ISO vs. WT-ISO, *: $P=1.31E-05$ KO+ISO vs. KO-ISO, #: $P=0.0357$ KO+ISO vs. WT+ISO, $n=15-28$ cells from 4-5 mice) in adult cardiomyocytes from WT or KO mice after 4-week ISO administration. For **K-M**, cells in +ISO groups were cultured with ISO (0.1 $\mu\text{mol/L}$) for 2 days for indicator expression. For **N**, freshly isolated cells were used. **O-Q**, Effects of BAPTA-AM (0.1 $\mu\text{mol/L}$, **O-P**) or calpain inhibitor XI (Cal XI, 1 $\mu\text{mol/L}$, **Q**) on ISO (0.1 $\mu\text{mol/L}$, 24 hr)-induced caspase 3/7 activity (**O**, **Q**) or calpain1/2 activity (**P**) in adult cardiomyocytes from KO mice. In **O**, *: $P=7.723E-08$ +ISO vs. -ISO, *: $P=7.01E-05$ BAPTA+ISO vs. BAPTA-ISO. In **P**, *: $P=0.0019$ +ISO vs. -ISO. In **Q**, *: $P=1.58E-08$ +ISO vs. -ISO, *: $P=0.0004$ BAPTA+ISO vs. BAPTA-ISO. $n=4$. **R**, Calpain1/2 activity in mouse heart. *: $P=0.0007$ KO+ISO vs. KO-ISO, #: $P=0.0357$ KO+ISO vs. WT+ISO. $n=5$ for -ISO groups and $n=6-8$ for +ISO groups.

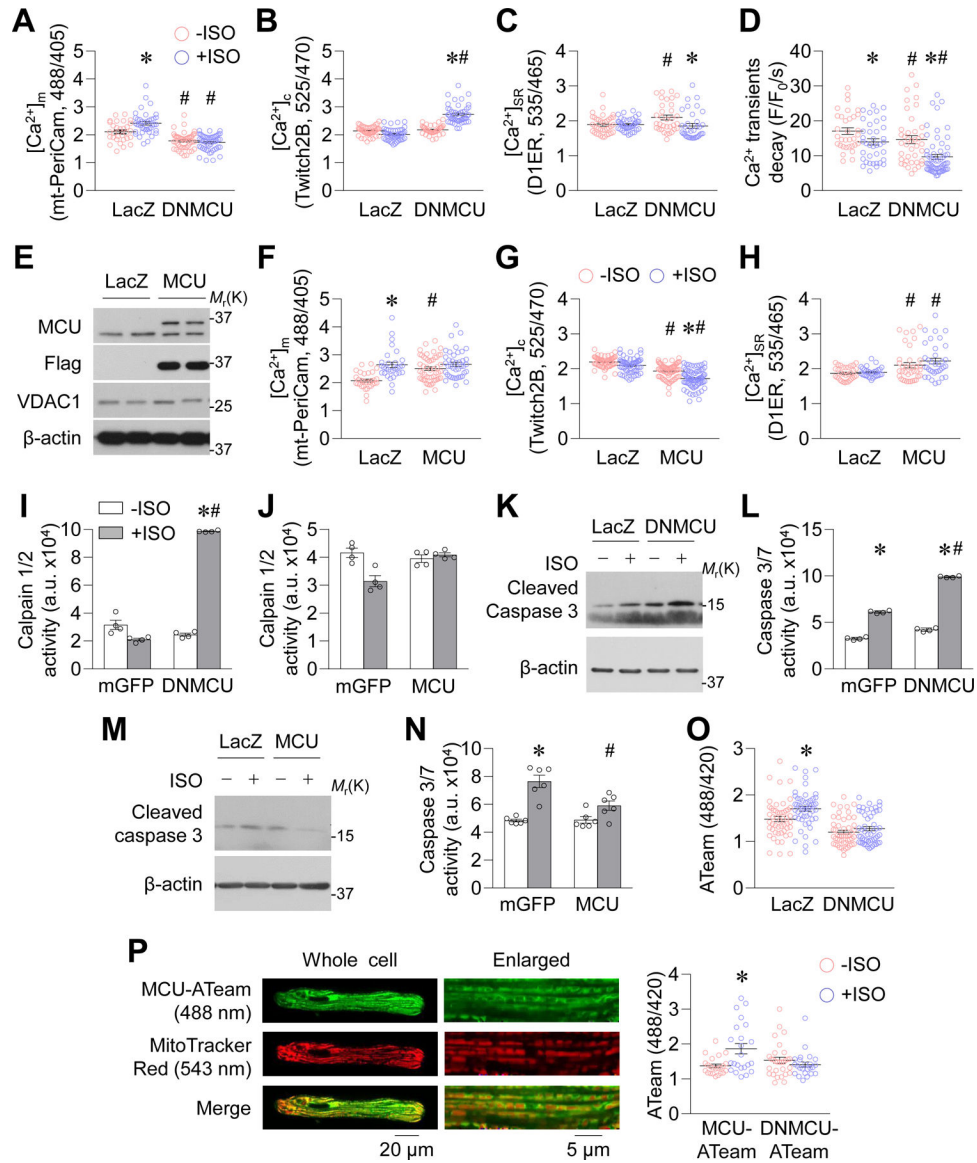


Figure 3. MCU modulated Ca^{2+} and energy homeostasis in rat cardiomyocytes during chronic ISO incubation.

A-D, Mitochondrial Ca^{2+} concentrations (**A**, $[Ca^{2+}]_m$, measured by mt-PeriCam, *: $P=0.0013$ LacZ+ISO vs. LacZ-ISO, #: $P=2.979E-05$ DNMCU-ISO vs. LacZ-ISO, #: $P=2E-15$ DNMCU+ISO vs. LacZ+ISO, $n=48-71$ cells from 5-7 rats), cytosolic Ca^{2+} concentrations (**B**, $[Ca^{2+}]_c$, measured by Twitch2B, *: $P=8.32E-12$ DNMCU+ISO vs. DNMCU-ISO, #: $P=2.71E-23$ DNMCU+ISO vs. LacZ+ISO, $n=36-69$ cells from 4-7 rats), SR Ca^{2+} content (**C**, $[Ca^{2+}]_{SR}$, measured by D1ER, #: $P=0.0121$ DNMCU-ISO vs. LacZ-ISO, *: $P=0.0155$ DNMCU+ISO vs. DNMCU-ISO, $n=33-41$ cells from 4 rats), and the decay rate of pacing-induced cytosolic Ca^{2+} transients (**D**, measured by GCaMP6f, *: $P=0.0202$ LacZ+ISO vs. LacZ-ISO, #: $P=0.0113$ DNMCU-ISO vs. LacZ-ISO, *: $P=0.0002$ DNMCU+ISO vs. DNMCU-ISO, #: $P=0.0003$ DNMCU+ISO vs. LacZ+ISO, $n=33-60$ cells from 5 rats) in adult rat cardiomyocytes overexpressing dominant negative MCU (DNMCU) and incubated with ISO (0.1 μ mol/L, 24 hr). **E**, Western blotting images showing the

protein levels of overexpressed flag-tagged MCU in adult rat cardiomyocytes. **F-H**, $[Ca^{2+}]_m$, $[Ca^{2+}]_c$, and $[Ca^{2+}]_{SR}$ in adult rat cardiomyocytes overexpressing MCU and with ISO (0.1 $\mu\text{mol/L}$, 24 hr) incubation. In **F**, *: $P=3.86E-06$ LacZ+ISO vs. LacZ-ISO, #: $P=0.0964$ MCU+ISO vs. LacZ+ISO, $n=48-71$ cells from 5-7 rats. In **G**, #: $P=1.5E-05$ MCU-ISO vs. LacZ-ISO, *: $P=8.9E-08$ MCU+ISO vs. MCU-ISO, #: $P=3.27E-16$ MCU+ISO vs. LacZ+ISO, $n=36-69$ cells from 4-7 rats. In **H**, #: $P=0.0238$ MCU-ISO vs. LacZ-ISO, #: $P=0.0006$ MCU+ISO vs. LacZ+ISO, $n=33-41$ cells from 4 rats. **I-J**, Calpain 1/2 activity in adult rat cardiomyocytes. In **I**, *: $P=8.4E-10$ DNMCU+ISO vs. DNMCU-ISO, #: $P=2.70E-10$ DNMCU+ISO vs. mGFP+ISO, $n=4$. For **J**, $n=6$. **K-N**, Cleaved caspase 3 (**K, M**) and caspase 3/7 activity (**L, N**) in adult rat cardiomyocytes. $n=3$ for **K** and **M**. In **L**, *: $P=1.13E-07$ mGFP+ISO vs. mGFP-ISO, *: $P=2.44E-09$ DNMCU+ISO vs. DNMCU-ISO, #: $P=6.89E-09$ DNMCU+ISO vs. mGFP+ISO, $n=4$. In **N**, *: $P=0.0077$ mGFP+ISO vs. mGFP-ISO, #: $P=0.0232$ MCU+ISO vs. mGFP+ISO, $n=6$. **O**, Cytosolic ATP levels measured by ATeam in adult rat cardiomyocytes. *: $P=0.0048$ LacZ+ISO vs. LacZ-ISO, $n=51-53$ cells from 5 rats. For **A-O**, LacZ or mGFP was expressed in Control cells. **P**, Representative confocal images and summarized data showing mitochondrial ATP measurements in adult rat cardiomyocytes overexpressing MCU-ATeam or DNMCU-ATeam and with ISO incubation. *: $P=0.003$ MCU-ATeam +ISO vs. MCU-ATeam -ISO, $n=23-30$ cells from 4-6 rats.

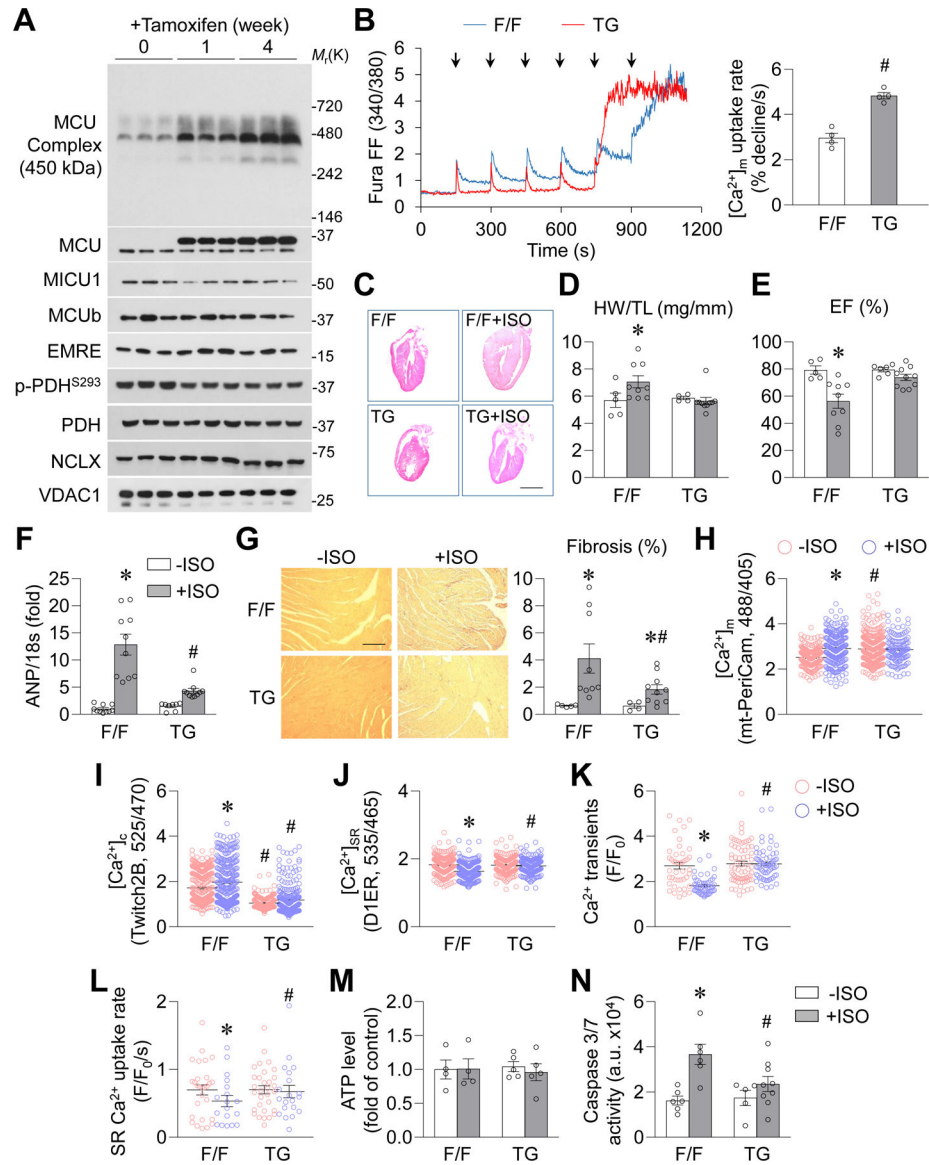


Figure 4. MCU TG ameliorated heart dysfunction induced by chronic β -AR activation.
A, Time-dependent changes in MCU, MCU complex, MCU regulatory proteins, NCLX and PDH phosphorylation in cardiac mitochondria from MCU floxed/floxed (F/F) mice after tamoxifen induction. $n=3$. **B**, Representative traces and normalized data showing Ca^{2+} uptake by freshly isolated mitochondria from the heart of MCU TG (TG) or F/F mouse. #: $P=0.0003$ TG vs. F/F. $n=4$. Arrows indicate the additions of Ca^{2+} (25 $\mu\text{mol/L}$ each time). **C**, Representative H&E staining images of the coronal section of whole heart from F/F or TG mouse after ISO administration (10 mg/kg/day, 4 weeks). Scale bar = 5 mm. **D**, Heart weight to tibia length ratio (HW/TL) in F/F or TG mouse. *: $P=1.13\text{E-}07$ F/F+ISO vs. F/F-ISO. $n=5$ for -ISO groups and $n=9-10$ for +ISO groups. **E**, Left ventricular ejection fraction in F/F or TG mouse. *: $P=0.0087$ F/F+ISO vs. F/F-ISO. $n=5-6$ for -ISO groups and $n=9-10$ for +ISO groups. **F**, mRNA level of ANP in mouse heart. *: $P=8.76\text{E-}06$ F/F+ISO vs. F/F-ISO, #: $P=0.0005$ TG+ISO vs. F/F+ISO. $n=8-10$. **G**, Picrosirius red staining for fibrosis

in mouse heart. *: P=0.0357 F/F+ISO vs. F/F-ISO, *: P=0.0499 TG+ISO vs. TG-ISO, #: P=0.0364 TG+ISO vs. F/F+ISO. n=5-6 for -ISO groups and n=9-10 for +ISO groups. Scale bar = 100 μm . **H-L**, Mitochondrial Ca^{2+} concentrations (**H**, $[\text{Ca}^{2+}]_{\text{m}}$, measured by mt-PeriCam, *: P=1.84E-05 F/F+ISO vs. F/F-ISO, #: P=0.0263 TG-ISO vs. F/F-ISO, n=132-329 cells from 4-5 mice), cytosolic Ca^{2+} concentrations (**I**, $[\text{Ca}^{2+}]_{\text{c}}$, measured by Twitch2B, *: P=0.0232 F/F+ISO vs. F/F-ISO, #: P=5.64E-11 TG-ISO vs. F/F-ISO, #: P=2.79E-06 TG+ISO vs. F/F+ISO, n=191-243 cells from 4-5 mice), SR Ca^{2+} content (**J**, $[\text{Ca}^{2+}]_{\text{SR}}$, measured by D1ER, *: P=3.18E-09 F/F+ISO vs. F/F-ISO, #: P=0.0002 TG+ISO vs. F/F+ISO, n=162-257 cells from 4-5 mice), pacing-induced cytosolic Ca^{2+} transients (**K**, fluo-8, *: P=1.95E-08 F/F+ISO vs. F/F-ISO, #: P=1.62E-17 TG+ISO vs. F/F-ISO, n=53-74 cells from 4-5 mice), and SR Ca^{2+} uptake rate (**L**, *: P=0.0265 F/F+ISO vs. F/F-ISO, #: P=0.0424 TG+ISO vs. F/F-ISO, n=22-36 cells from 4 mice) in adult cardiomyocytes from F/F or MCU TG mouse after 4-week ISO administration. For **H-K**, the cells in +ISO groups were cultured with ISO (0.1 $\mu\text{mol/L}$) for 2 days after isolation for indicator expression. For **L**, freshly isolated cells were used. **M**, ATP levels measured in whole heart homogenates. n=4-5. **N**, Caspase 3/7 activity in mouse hearts. *: P=0.0019 F/F+ISO vs. F/F-ISO, #: P=0.0319 TG+ISO vs. F/F+ISO. n=5-9.

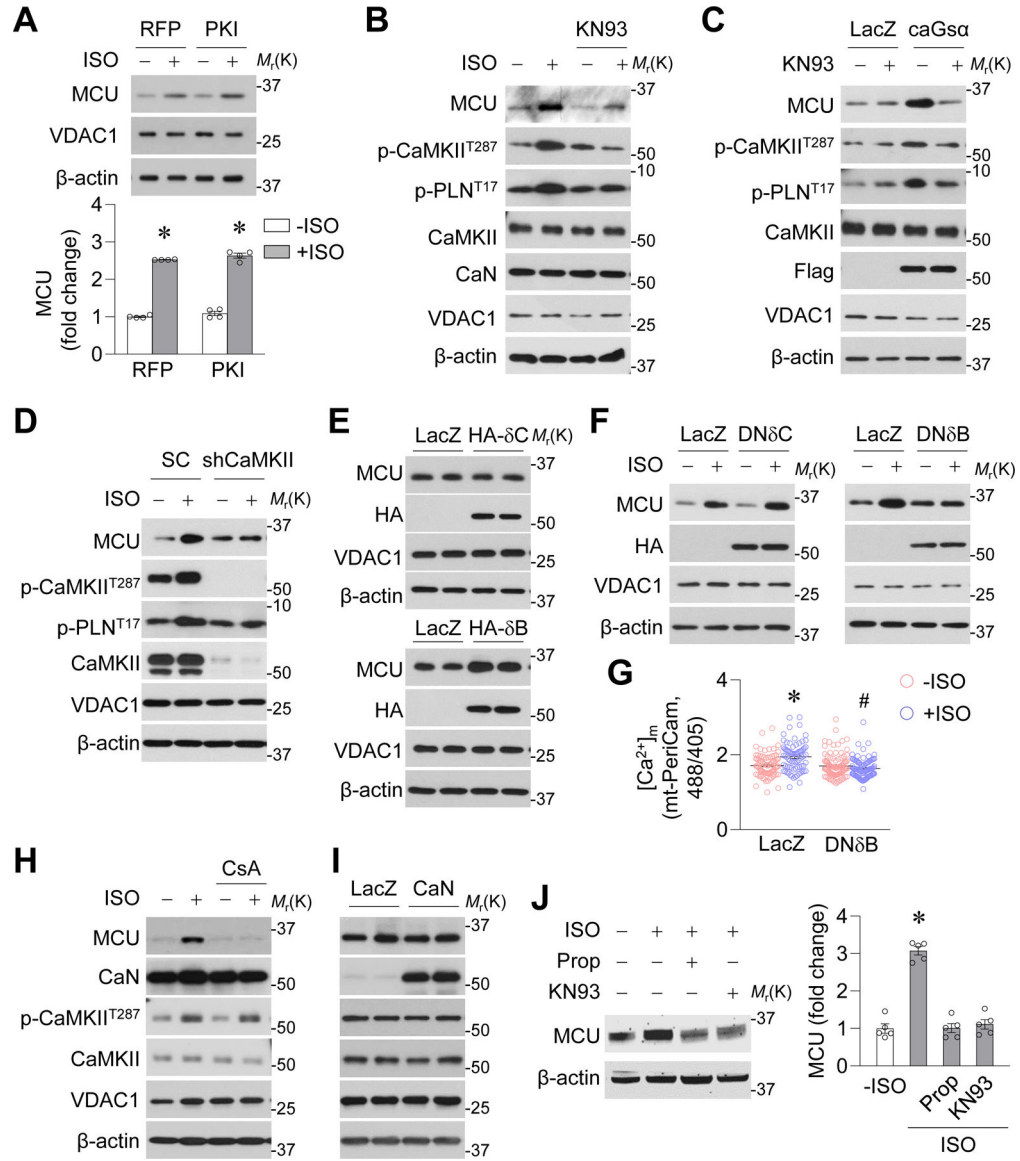


Figure 5. Chronic β -AR activation upregulated MCU through CaMKII δ B.

A-B, Effects of PKA inhibitory peptide (PKI, **A**, *: $P=2.72E-10$ RFP+ISO vs. RFP-ISO, *: $P=2.04E-06$ PKI+ISO vs. PKI-ISO) or CaMKII inhibitor (KN93, 0.5 μ mol/L, **B**) on ISO (0.1 μ mol/L, 24 hr) induced MCU upregulation in adult mouse cardiomyocytes. $n=4$. **C**, Effects of KN93 on constitutively active Gs α (caGs α) induced MCU upregulation. $n=4$. **D**, Effects of CaMKII δ shRNA (shCaMKII) on ISO-induced MCU upregulation. $n=4$. **E**, Effects of overexpressing HA-tagged CaMKII δ C (δ C) or δ B on MCU expression. $n=4$. **F**, Effect of overexpressing HA-tagged dominant negative CaMKII δ C (DN δ C) or DN δ B on ISO-induced MCU upregulation. $n=4$. **G**, Effects of DN δ B on mitochondrial Ca²⁺ concentrations (measured by mt-PeriCam) after ISO administration. *: $P=3.04E-05$ LacZ+ISO vs. LacZ-ISO, #: $P=4.89E-10$ DN δ B+ISO vs. LacZ+ISO. $n=79-98$ cells from 4 mice. **H**, Effects of calcineurin (CaN) inhibitor cyclosporine A (CsA, 1 μ mol/L) on ISO-induced MCU upregulation. $n=4$. **I**, Effects of CaN overexpression on MCU protein

levels. n=4. **J**, Effects of β blocker propranolol (Prop, 10 mg/kg/day) or KN93 (10 μ mol/kg/day) on ISO-induced MCU upregulation in mouse hearts *: P=1.529E-06 +ISO vs. -ISO. n=6.

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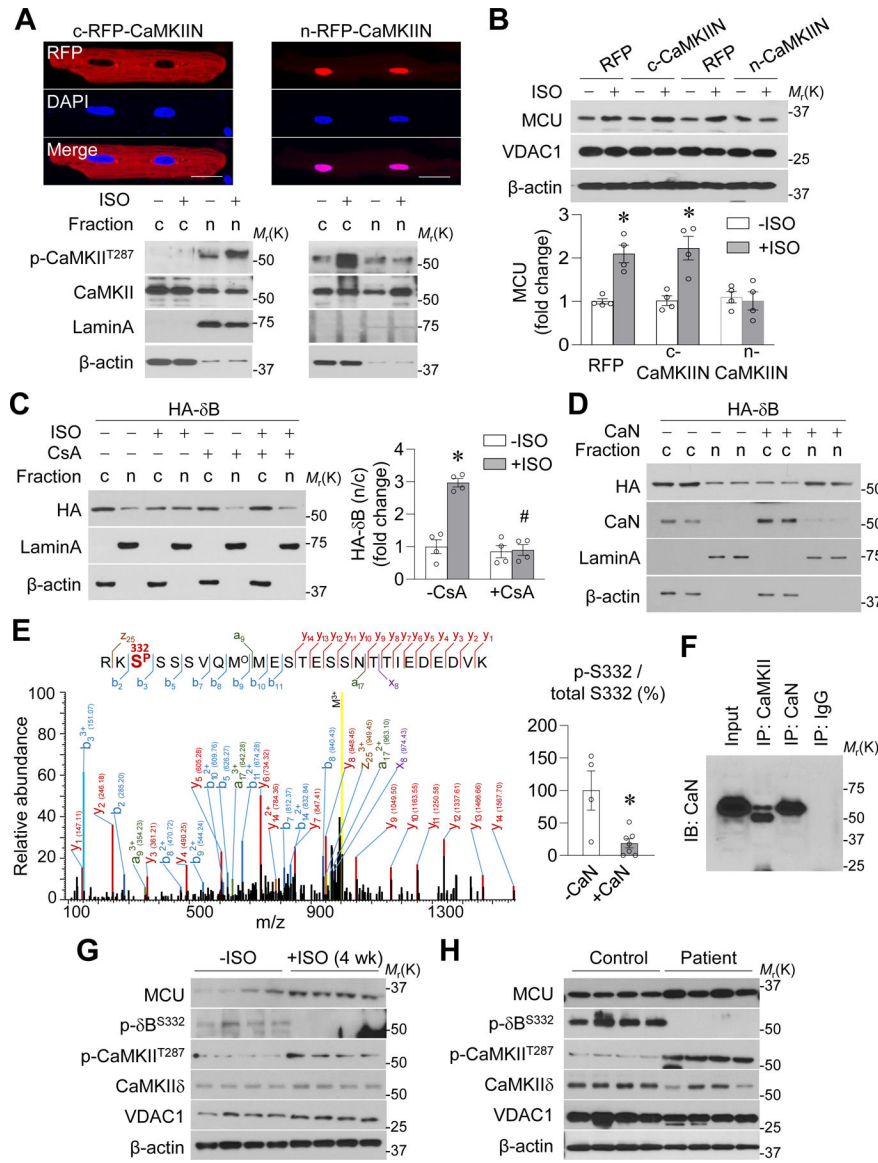


Figure 6. Chronic β -AR activation promoted nuclear translocation of CaMKII δ B via calcineurin-mediated S332 de-phosphorylation.
A-B, Effects of overexpressing cytosol-targeted and RFP-tagged CaMKII inhibitory protein (c-CaMKIIN) or nucleus-targeted CaMKIIN (n-CaMKIIN) on CaMKII T287 phosphorylation in cytosol or nucleus (**A**) and on ISO-induced MCU upregulation (**B**) in adult mouse cardiomyocytes. *: $P=0.002$ RFP+ISO vs. RFP-ISO, *: $P=0.0061$ c-CaMKIIN+ISO vs. c-CaMKIIN-ISO. $n=4$. **C**, Effects of ISO (0.1 $\mu\text{mol/L}$) and cyclosporine A (CsA, 0.1 $\mu\text{mol/L}$) on intracellular location of HA-tagged CaMKII δ B (δ B) in adult mouse cardiomyocytes. *: $P=0.0002$ +ISO vs. -ISO, #: $P=5.859\text{E-}05$ ISO+CsA vs. ISO-CsA. $n=4$. **D**, Effects of overexpressing calcineurin (CaN) on intracellular location of δ B in adult cardiomyocytes. $n=4$. **E**, Representative mass spectrum of a peptide in CaMKII δ B containing S332 phosphorylation. The peptide sequence and phosphorylation site were confidently identified based on the matched b- or a- and y- or x- ion series indicated by the spectrum annotations. Right panel showed that the abundance of peptide

containing phosphorylated S332 was significantly lower after CaN incubation. *: $P=0.0077$ +CaN vs. -CaN. n=4–6. **F**, Co-IP showing the binding between CaMKII δ B and CaN in adult cardiomyocytes. n=3. **G-H**, Western blotting images showing the detection of δ B phosphorylation at S332 or T287 and MCU protein level in mouse hearts after ISO administration for 4 weeks (**G**) or in human failing heart samples (**H**). n=4.

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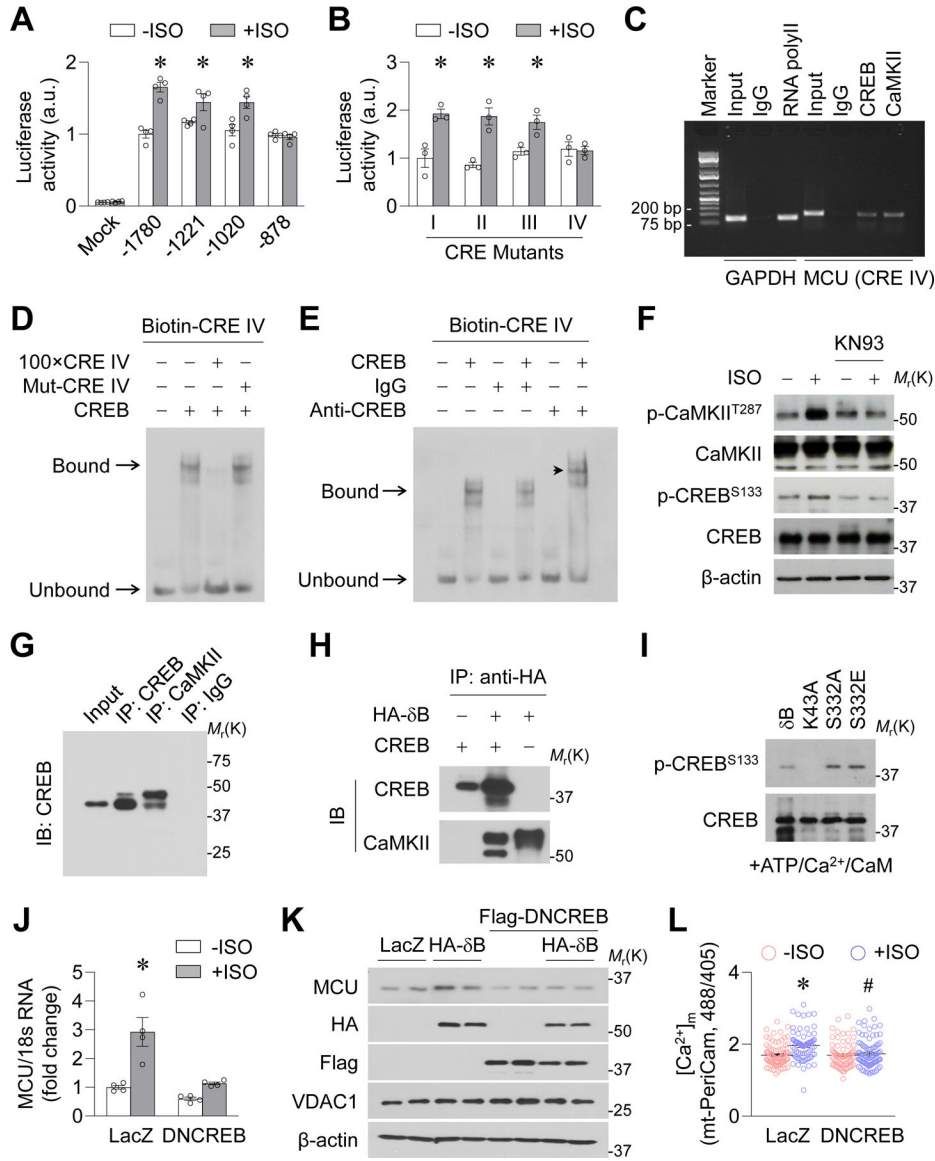


Figure 7. CaMKII δ B phosphorylated CREB to modulate MCU gene expression.
A-B, Luciferase activity assay showing the activity of truncated MCU promoter (**A**, *: P=0.0003, 0.0149 or 0.0414 +ISO vs. -ISO for -1780, -1221 or -1020 groups, respectively) or the MCU promoter fragments that contain mutated sequences of CRE I-IV (**B**, *: P=0.0131, 0.0053 or 0.022 +ISO vs. -ISO for I, II or III groups, respectively) in H9C2 myoblasts. n=4. **C**, Chromatin immunoprecipitation assay showing the binding of CRE IV with CREB and CRE IV with CaMKII in adult cardiomyocytes. The binding between RNA polymerase II and a sequence in GAPDH promoter was used as positive control. n=3. **D**, Electrophoretic mobility shift assay (EMSA) showing the binding between CREB and double-stranded and biotin-labeled oligonucleotides corresponding to CRE IV sequence (Biotin-CRE IV) *in vitro*, which was blocked by 100 fold unlabeled CRE IV sequence (100xCRE IV) but not by mutated biotin-CRE IV (Mut-CRE IV). **E**, EMSA showing that addition of CREB antibody caused a shift of the band toward higher molecular

weight (arrow head). n=3. **F**, Effects of KN93 on ISO-induced CREB phosphorylation at S133 in adult mouse cardiomyocytes. n=4. **G**, Co-IP showing the binding between endogenous CaMKII and CREB in adult mouse cardiomyocytes. Representative of 4 repeats. **H**, Pull down analysis showing the binding between HA-CaMKII δ B and CREB *in vitro*. Representative of 3 repeats. **I**, *In vitro* phosphorylation assay showing the effects of wild type (δ B), S332A, S332E or K43A mutations of CaMKII δ B on CREB phosphorylation at S133. Representative of 3 repeats. **J**, Effects of dominant negative CREB (DNCREB) on ISO-induced MCU mRNA upregulation in adult mouse cardiomyocytes. *: P=0.0087 LacZ+ISO vs. LacZ-ISO. n=6. **K**, Effects of DNCREB on HA- δ B-induced MCU upregulation. n=4. **L**, Effects of DNCREB on ISO-induced changes in mitochondrial Ca²⁺ concentrations ([Ca²⁺]_m, measured by mt-PeriCam. *: P=5.440E-06 LacZ+ISO vs. LacZ-ISO. #: P=0.0003 DNCREB+ISO vs. LacZ+ISO, n=76-93 cells from 4 mice.

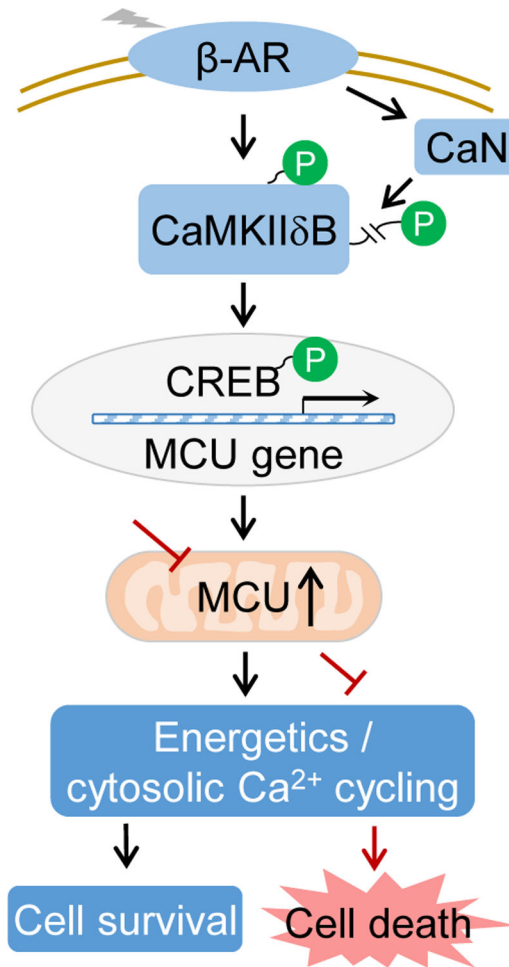


Figure. 8. Schematic illustration.

MCU expression is upregulated by the β -AR/CaMKII δ B/CREB pathway to maintain cytosolic Ca^{2+} and energy homeostasis in the heart. Phosphorylation at T287 increased the kinase activity of CaMKII δ B and de-phosphorylation at S332 triggered its nuclear translocation, both of which are needed for MCU gene upregulation.