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Original Article

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Phosphorylation in Novel Mitochondrial Creatine Kinase Tyrosine Residues Render Cardioprotection against Hypoxia/Reoxygenation Injury

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ABSTRACT

Objective: Ischemic cardiomyopathy (ICM) is the leading cause of heart failure. Proteomic and genomic studies have demonstrated ischemic preconditioning (IPC) can assert cardioprotection against ICM through mitochondrial function regulation. Considering IPC is conducted in a relatively brief period, regulation of protein expression also occurs very rapidly, highlighting the importance of protein function modulation by post-translational modifications. This study aimed to identify and analyze novel phosphorylated mitochondrial proteins that can be harnessed for therapeutic strategies for preventing ischemia/reperfusion (I/R) injury.
Methods: Sprague-Dawley rat hearts were used in an *ex vivo* Langendorff system to simulate normal perfusion, I/R, and IPC condition, after which the samples were prepared for phosphoproteomic analysis. Employing human cardiomyocyte AC16 cells, we investigated the cardioprotective role of CKMT2 through overexpression and how site-directed mutagenesis of putative CKMT2 phosphorylation sites (Y159A, Y255A, and Y368A) can affect cardioprotection by measuring CKMT2 protein activity, mitochondrial function and protein expression changes.
Results: The phosphoproteomic analysis revealed dephosphorylation of mitochondrial creatine kinase (CKMT2) during ischemia and I/R, while preserving its phosphorylated state during IPC. CKMT2 overexpression conferred cardioprotection against hypoxia/reoxygenation (H/R) by increasing cell viability and mitochondrial adenosine triphosphate level, preserving mitochondrial membrane potential, and reduced reactive oxygen species (ROS) generation, while phosphomutations, especially in Y368, nullified cardioprotection by significantly reducing cell viability and increasing ROS production during H/R. CKMT2 overexpression increased mitochondrial function by mediating the proliferator-activated receptor γ coactivator-1α/estrogen-related receptor-α pathway, and these effects were mostly inhibited by Y368A mutation.
Conclusion: These results suggest that regulation of quantitative expression and phosphorylation site Y368 of CKMT2 offers a unique mechanism in future ICM therapeutics.

Keywords: Creatine kinase, mitochondrial form; Hypoxia; Reoxygenation; Mitochondria; Phosphorylation

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Conflict of Interest

The authors have no conflict of interest to declare.

Author Contributions

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INTRODUCTION

Ischemic cardiomyopathy (ICM) occurs when the lack of adequate blood flow to the cardiomyocytes results in the heart muscles not being able to meet its electric, functional, and metabolic needs, effectively reducing the pumping ability of the heart.[1] ICM is the leading cause of heart failure globally yet mortality rates remain substantially high despite scientific advances.[2] Effects of ICM can be alleviated with the use of ischemic preconditioning (IPC), which are quick bouts of ischemia before a prolonged ischemia/reperfusion (I/R) injury.[3] As such, there have been growing calls to look beyond conventional cardioprotective methods in addressing ICM, and rather identify new targets regulated during IPC such as the mitochondria when considering approaches to address the disease.[4,5]

Mitochondria are deeply implicated in the progression of cardiac diseases, making it an attractive target for ICM therapeutic strategies. Mitochondrial reprogramming and dysfunction and damage to its ultrastructure have long been hallmarks of a pathological heart.[6] Modern technology has allowed researchers to profile the cardiac genome and proteome that made it easier to identify target proteins and understand previously unknown mechanisms related to cardiac pathology. Proteomic and genomic studies were able to ascertain the critical role of metabolic processes such as oxidative phosphorylation, cardiac metabolism, and protein folding in the progression of ICM and heart failure.[7,8] As such, several proteomic studies in I/R samples have determined post-translationally altered cardiac mitochondrial proteins.

Protein post-translational modifications are essential for understanding cardiovascular diseases, considering these alterations after the completion of the translational process can regulate a plethora of other functions and factors not only in physiological processes but also in pathology.[9] proteomic studies have identified proteins that are posttranslationally altered during ischemia. However, the downside to these phosphoproteomic studies is that most of these proteins do not necessarily focus on the cardiac mitochondrial proteome and/or have not been functionally studied and validated experimentally. Thus, we conducted our own phosphoproteomic analysis of cardiac mitochondrial proteins posttranslationally altered during perfusion, I/R, and IPC condition to identify novel proteins that can be targeted for cardiac protection. One of the candidates that stood out was CKMT2.

The mitochondrial creatine kinase (CKMT2), one of the creatine kinase (CK) isoenzymes, is tightly coupled to adenosine triphosphate (ATP) export via adenine nucleotide transporter or carrier, making it an important player in ATP-synthesis and respiratory chain activity.[10,11] CKMT2 activity is correlated to oxidative capacity as it increases the availability of ADP for complex V of the respiratory chain, thus regulating mitochondrial membrane potential ($\Delta\psi m$) and reactive oxygen species (ROS) formation.[11,14] CKMT2 has previously been implicated in various cardiac dysfunction. Under hypoxic conditions, an increase in CK activity can be attributed mainly to increased CKMT2 as an adaptive response to improve oxidative phosphorylation.[15-17] Its modest elevation in the heart do not pose any unfavorable effects to cellular metabolism, mitochondrial or *in vivo* cardiac function, but regulates mitochondrial permeability transition pore opening to protect against I/R injury and ameliorate functional recovery. Enhancing CKMT2 expression thus can possibly provide an important strategy for improving myocardial ATP delivery and mechanical function for reducing I/R injury. However, phosphorylation of CKMT2 in both physiological and pathological states have not yet been studied.

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Here we first characterized how CKMT2 overexpression renders protection during hypoxia/reoxygenation (H/R). We also determined vital phosphorylation sites in CKMT2 and described how its phosphorylation can affect mitochondrial function which can lend cardiac protection. For this purpose, we used transient expression in AC16 cell line. The current study illustrates the essential phosphorylation sites in CKMT2, especially Y368, and how these sites mediate cardiac protection against H/R injury through mitochondrial function and protein regulation, offering new targets and insights in developing future therapeutic strategies for ICM.

MATERIALS AND METHODS

1. Patients

Patients were categorized as non-responders when their left-ventricular (LV) ejection fraction did not show more than 10% improvement at remote phase after biopsy. Consent for biopsy procedures or use of explanted tissues prospectively were obtained in all cases. Study procedures involving humans were approved by the Research Ethic Committee of Niigata University.

2. Ethics statement

All experimental procedures in animals in this study were approved by the Institutional Review Board of Animals, Inje University College of Medicine (approval No. 2011-049). Prior to surgery, all animals were anaesthetized using sodium pentobarbital to minimize animal suffering.

3. Langendorff perfusion of isolated rat hearts

Eight-week Sprague-Dawley rats were anaesthetized intraperitoneally (100 mg/kg) before sacrifice, and was checked for stimuli response after to confirm anaesthetization. Rat hearts were excised surgically and quickly mounted onto the Langendorff system and perfused with normal Tyrode's solution to remove all blood. Stabilization was performed for 20 minutes before conducting the experiments. Control hearts were continuously perfused for 40 minutes. Ischemia only was performed for 20 minutes. I/R hearts were exposed to 20 minutes of ischemia, followed by 20 minutes of reperfusion. IPC of hearts was performed for total of 20 minutes, followed by 20 minutes of perfusion.[18,19]

4. Cardiac sample preparation and phosphoproteomics

Whole heart samples were isolated and prepared as previously described.[20] Samples were sent to Bioconvergence (Suwon, Korea) and were then analyzed via 2-dimensional electrophoresis and matrix-associated laser desorption ionization time of flight mass spectrometry analysis. Methodology employed were similar to a previously described paper.[19] The Gene Ontology (GO) enrichment analysis was carried out using Protein Analysis Through Evolutionary Relationships (PANTHER; http://www.pantherdb.org/about.jsp) and Database for Annotation, Visualization and Integrated Discovery (DAVID; https://david.ncifcrf.gov).[21-23] Protein-protein interactions were visualized using Search Tool for the Retrieval of Interacting Gene Database (STRING, https://string-db.org/cgi/about.pl) version 11.0 with a confidence cutoff of 0.4.[24]

5. *In silico* analysis

Phosphorylation sites were primarily identified through PhosphoSitePlus in *Homo sapiens*, *Mus musculus*, and *Rattus norvegicus* data, where modifications in residues were recorded through proteomic discovery mass spectrometry. Tyr^{159} (Y159), Tyr^{255} (Y255), and Tyr^{368}

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(Y368), the 3 residues recorded to have the most hits were then analyzed and calculated for its conservation across 150 different species using ConSurf server.[25] HOPE mutant analysis server was then used to determine deleterious effects of protein mutation at selected residues.[26] CKMT2 structure was retrieved from PDB database (CKMT ID: 4Z9M) and was rendered using Biorender.

6. Plasmid constructs and site-directed mutagenesis

Full-length CKMT2 (GenBank Accession No. NM_001099735.2) was created using a complementary DNA template by polymerase chain reaction (PCR). A site-directed mutagenesis system (EZ024S; Enzynomics, Daejeon, Korea) was used to mutate the constructs of Y159A, Y255A, Y368A, and Y159AY255AY368A. The resulting PCR products were cloned into GFPN1 and pcDNA6.C vectors using In-Fusion® HD Cloning Kit (102518; Takara Bio, Mountain View, CA, USA).

7. Cell culture and transfection

AC16 cells were cultured in DMEM/F-12 (12634028; Gibco, Carlsbad, CA, USA) media with 10% FBS (12484028; Gibco) and 100 units/ml penicillin-streptomycin (15140-122; Gibco) at 37°C with 5% CO2. Transfection of expression vectors was performed using TurboFect™ Transfection Reagent (R0532; Thermo Fisher Scientific, Hanover Park, IL, USA) according to the manufacturer's protocol.

8. Simulated H/R conditions

Equal numbers of cells (2.4×10^5 cells) were plated onto 6-well tissue culture plates. After 24 hours, GFPN1–CKMT2 and GFPN1-mutant plasmids were transfected into AC16 cells and subjected to 1% O_2, in hypoxia condition for 18 hours, followed by 2 hours of reoxygenation at 18% O_2, 5% CO_2, at 37°C under normoxia. Cells were collected and resuspended in phosphate-buffered saline (PBS) solution before analysis.

9. Localization and confocal microscopy

AC16 cells were transfected with pGFPN1-CKMT2 and mutant constructs and were visualized under a confocal microscope (LSM-700; Carl Zeiss, Oberkochen, Germany). For the detection of $\Delta\psi m$, cells were stained with 200 nM TMRE for 30 minutes at 37°C.

10. Measurement of Cell viability

Equal numbers of cells (1.2×10^4 cells/well) were plated onto 96-well tissue culture plates and incubated the plate in a humidified incubator (5% CO_2, at 37°C) for 24 hours. 10 μL of the CCK8 solution (CK04-20; Dojindo Molecular Technologies, Kumamoto, Japan) were added to each well and then incubated for 4 h. Cells were measured for their absorbance at 450nm using SpectraMax M3 microplate reader (Molecular Devices, San Jose, CA, USA).

11. Measurement of mitochondria ATP content

Equal numbers of cells (1.2×10^4, 96-well tissue culture plates) were lysed to determine mitochondria ATP using an ATP detection kit (G7573; Promega, Madison, WI, USA) according to the manufacturer's protocol. Chemiluminescence was measured using SpectraMax M3 microplate reader (Molecular Devices). ATP production independent of oxidative phosphorylation was determined by treating cells with 1 μM rotenone (R8875; Sigma Aldrich, St. Louis, MO, USA) and 1 μM antimycin A (A8674; Sigma Aldrich) for 1 hour before collection. ATP production by oxidative phosphorylation was calculated by subtracting (ATP produced in cells treated with rotenone and antimycin A) from (total cellular ATP).

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12. Analysis of TMRE and mitochondria-ROS

GFPN1–CKMT2 and GFPN1-mutant plasmids were transfected into AC16 cells. AC16 cells were exposed to H/R conditions as explained above. After H/R, cells were stained with 200 nM TMRE (T669) or 1 μM MitoSOX™ Red Mitochondrial Superoxide Indicator (M36008; both from Thermo Fisher Scientific, Waltham, MA, USA) for 30 minutes at 37°C to measure levels of Δψm or superoxide production. After washing with PBS, cells were analyzed flow cytometry using FACS Calibur (BD Biosciences, Franklin Lakes, NJ, USA).

13. CKMT2 activity assay

One mL of CK-NAC (TR14110; Thermo Fisher Scientific) was incubated together with 20 μL of cell lysate was incubated at 30°C. Three minutes later, CK activity was measured using a CK activity assay kit (ab155091; Abcam, Cambridge, UK) according to the manufacturer's protocol and read using SpectraMax M3 microplate reader (Molecular Devices). The assay was performed in triplicate and results normalized to total protein levels using a BCA assay (23225; Thermo Fisher Scientific).

14. Purification of His-tagged protein

For His-tagged protein purify, pcDNA6.C/HIS, MYC-CKMT2 and pcDNA6.C/HIS, and MYC-mutant plasmids were transfected into HEK293 cells and extracted after 24 hours. Cell lysates of HIS-CKMT2 and mutant protein were purified using PRO·Hunt His·Bind Buffer Kit (EBE-1040; Elpisbio, Daejeon, Korea) following manufacturer protocol.

15. Western blot analysis

Proteins were extracted with RIPA buffer (50 mM Tris-HCL [pH 7.4], 150 mM NaCl, 1% Nonidet P-40, 0.2% sodium deoxycholate, 0.1% SDS) containing a protease inhibitor cocktail (11836170001; Roche, Penzberg, Germany). Proteins were then separated by SDS-PAGE and were transferred to a nitrocellulose membrane, which was blotted with antibodies against PGC1α (54481; Abcam), estrogen-related receptor-α (ERRα, ab93173; Abcam), glyceraldehyde 3-phosphate dehydrogenase (sc-32233; Santa Cruz Biotechnology, Dallas, TX, USA). Blots were then washed and incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies, followed by washing and immunoreactivity detection with enhanced chemiluminescence (RPN2232; GE Healthcare, Chicago, IL, USA).

16. Statistical analysis

Data were analyzed using unpaired Student's t-tests, 1-way or 2-way analysis of variance followed by Tukey's *post hoc* analysis wherever applicable. Values were expressed as mean±standard error of the mean, with p-values ≤0.05 considered statistically significant. Statistical analyses were carried out using GraphPad Prism 8.0 (GraphPad Software, Inc., San Diego, CA, USA).

RESULTS

1. Identification of cardiac protein phosphorylation and classification

Screening of candidate proteins were performed by comparing protein differential expression in rat hearts under perfusion (control), ischemia only, I/R, and IPC (**Fig. 1A**). Data analysis of cardiac proteins between perfused and IPC rat hearts identified a total of 871 phosphopeptides from a total of 265 proteins, 515 of which are exclusively phosphorylated during IPC only in 54 proteins (**Fig. 1B and C**). To determine which molecular functions were

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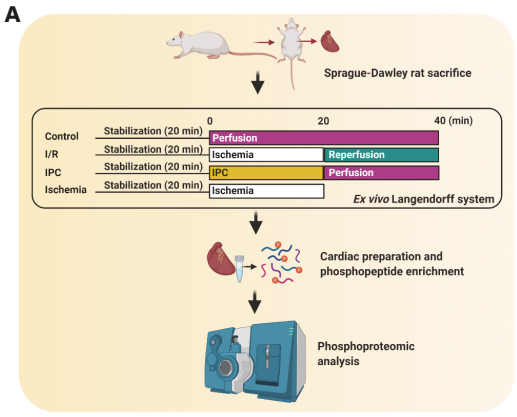
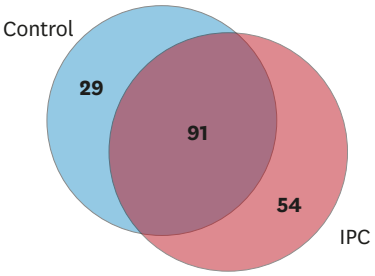


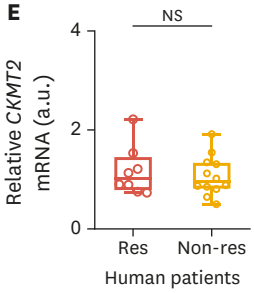
B

	Phosphoprotein	Phosphopeptide
Control	120	356
IPC	145	515



D

Protein	Condition	Amino acid sequence (phosphorylation sites in red)
Creatine Kinase S-type, Mitochondrial precursor [*Rattus norvegicus*], Accession number 189083744	Perfusion	**311** LGYILTCPSNLGTGLR **326** **355** GTGGVDTAAVADVYDISNIDR **375**
	Ischemia	**311** LGYILTCPSNLGTGLR **326** **355** GTGGVDTAAVADVYDISNIDR **375**
	I/R	**311** LGYILTCPSNLGTGLR **326** **355** GTGGVDTAAVADVYDISNIDR **375**
	IPC	**311** LGYILTCPSNLGTGLR **326** **355** GTGGVDTAAVADVYDISNIDR **375**



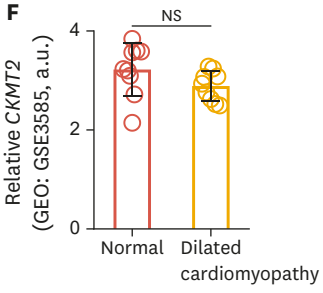


Fig. 1. Phosphoproteome analysis of cardiac proteins during control and IPC. (A) Schematic diagram illustrates the experimental workflow for rat heats, (B, C) after which total number of phosphopeptides are identified using liquid chromatography-tandem mass spectroscopy. (D) Phosphorylation of CKMT2 during perfusion and IPC compared to ischemia only and I/R. (E) CKMT2 mRNA level of healthy and non-responding human patients (responding n=12, non-responding n=8) (F) GEO data analysis (GSE3585) of CKMT2 mRNA in patients experiencing dilated cardiomyopathy (each group, n=9). Values are mean±standard error of the mean. (A, B) Two-tailed unpaired Student's *t*-tests.
I/R, ischemia/reperfusion; IPC, ischemic preconditioning; NS, statistically not significant; mRNA, messenger RNA.

altered during IPC, we performed a GO enrichment analysis of our gene list using PANTHER software against a *R. norvegicus* background. The enriched terms for molecular functions include protein binding, transcription regulation, and structural roles (**Supplementary Fig. 1A**). When checked for protein class, one of the enriched terms included metabolite interconversion (**Supplementary Fig. 1B**). These results were conferred with DAVID software using a *p*-value of 0.05, which rendered more specific terms. Enriched terms for biological processes included heart development and phosphorylation, few of which are located in the mitochondria and are involved in ATP binding (**Supplementary Fig. 1C-E**, **Supplementary Data 1**). Among the proteins phosphorylated only during IPC, only few fit most if not all of those aforementioned characteristics, one of which is CKMT2. Analysis using STRING also revealed significant upregulation in CKMT2 functional interaction with other phosphoproteins during IPC (**Supplementary Fig. 2A and B**).

2. CKMT2 messenger RNA (mRNA) remain unchanged in human cardiac disease models

Since we have identified a candidate protein CKMT2 based on phosphoproteomics, we investigated for a relationship between the expression of CKMT2 in patients and heart failure incidence. Patients were classified as non-responders when their LV ejection fraction did not show more than 10% improvement at the remote phase after biopsy. Non-responding patients exhibited no significant change in CKMT2 mRNA as compared to healthy patients (**Fig. 1E**), revealing a similar trend with patients experiencing dilated cardiomyopathy based on GEO database statistics (GSE3585) (**Fig. 1F**). Interestingly, data during perfusion and

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IPC of the rat hearts demonstrated that Y368 of CKMT2 is phosphorylated as compared to ischemia only and I/R, which displayed dephosphorylation at the same site. This can indicate that phosphorylation of Y368 can play an important role in cardioprotection (**Fig. 1D**, **Supplementary Table 1**). These data indicate a possible involvement of CKMT2 post-translationally during cardiac pathophysiology.

3. CKMT2 overexpression regulated mitochondrial function to confer cardioprotection during H/R

CKMT2 protein expression has been observed to be decreased in cardiac pathological models[15,27]; thus, CKMT2 overexpression can lead to recovery. To determine the effect of CKMT2 on mitochondria function and cell survival during a simulated I/R model, we performed *in vitro* analysis using AC16 human cardiac ventricular cell lines under normoxia or after 18 hours of hypoxia/2 hours reoxygenation (H/R) using MTS-GFP and CKMT2-GFP constructs (**Fig. 2A**). We first sought to establish mitochondrial localization of CKMT2 and determined its location in the mitochondrial inter membrane space, appearing in a red thread-like manner (**Fig. 2B**). We then proceeded to investigate whether AC16 cells transfected with MTS-GFP and CKMT2-GFP influence cell viability and intracellular ATP content under normoxia or H/R condition. Exposure of AC16 cells to H/R significantly decreased viability, but CKMT2-GFP overexpression renders protection as exhibited by a significant increase in cell survival (≤16% increase) as compared to MTS-GFP (**Fig. 2C**). ATP level in normoxia during CKMT2 overexpression was significantly increased, but not during H/R (**Fig. 2D**). ATP is not synthesized during hypoxia, and since CKMT2 is merely a

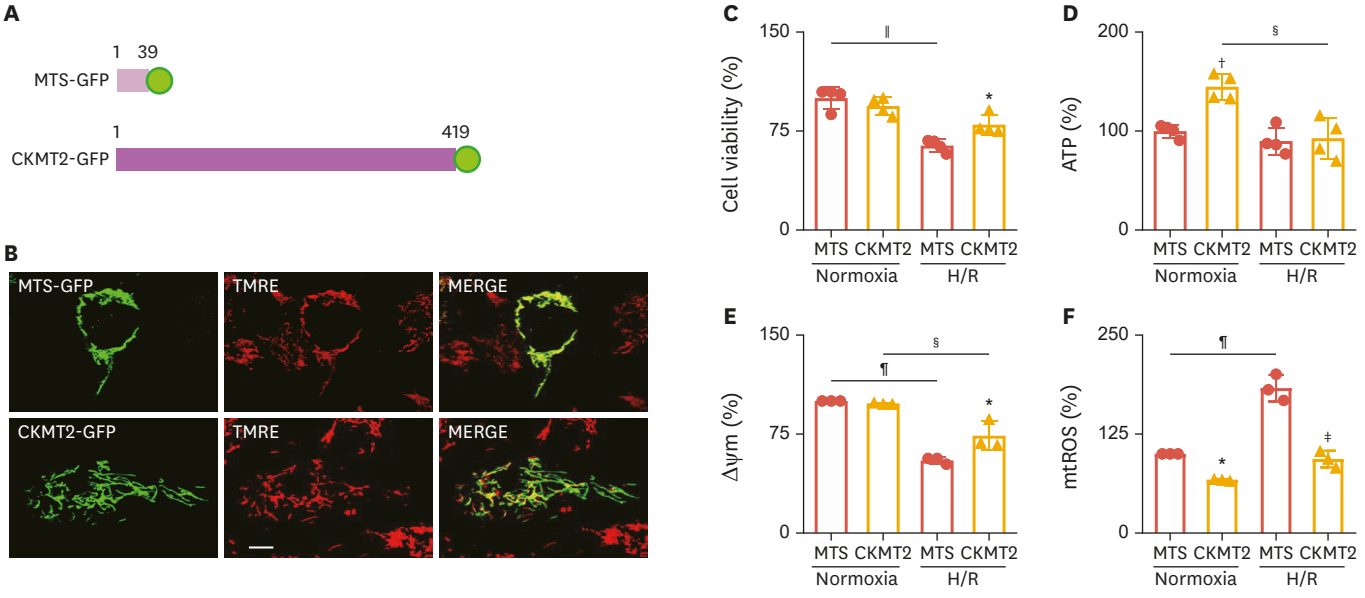

Fig. 2. CKMT2 overexpression protects against H/R in cardiac AC16 cells. (A) Schematic diagram of MTS control and CKMT2. (B) CKMT2-GFP and MTS-GFP were transfected into AC16 cells for 24 hours. verification of GFP-CKMT2 expression in transfected cells and co-localization with mitochondrial TMRE staining. Images were acquired by confocal microscopy, using a 60× objective. Green corresponds to GFP; red: TMRE; yellow: overlap of TMRE and CKMT2 within mitochondria (merged). Representative images are shown (scale bar=10 μm). (C, D) MTS-GFP and CKMT2-GFP were transfected into AC16 cells for 24 hours. Transfected cells were incubated in 2% O_2 for 18 hours to simulate hypoxia condition, followed by 2 hours re-oxygenation at 18% O_2, before conducting cell viability and intracellular ATP content analysis. (E, F) MTS-GFP vector and CKMT2 were transfected into AC16 cells for 24 hours. After hypoxia/re-oxygenation, cells were stained with 200 nM TMRE and 1 μM Mito-SOX for 30 minutes and analyzed by flow cytometry. Data were normalized to MTS-GFP. The data are expressed as the mean±standard error of the mean of 3 to 4 independent experiments, where applicable. (C-F) Two-way analysis of variance, *post hoc* Tukey's multiple comparisons test.
MTS, mitochondria target sequence; H/R, hypoxia/reoxygenation; ATP, adenosine triphosphate; Δψm, mitochondrial membrane potential; mtROS, mitochondrial reactive oxygen species.
*p<0.01, †p<0.01, ‡p < 0.0001 for intra-subgroup comparison; §p<0.01, ‖p<0.001, ¶p<0.0001 for inter-subgroup comparison.

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"converter" for the Cr/PCr shuttle, overexpression of CKMT2 did not affect ATP production because there are no metabolites to shuttle.

During hypoxic conditions, Δψm decreases below its endogenous steady-state level. This explains the decrease of Δψm during H/R in MTS-GFP, which ultimately recovered during CKMT2-GFP overexpression (≤19% higher vs MTS-GFP) to levels similar to normoxia (**Fig. 2E**). Recovery was also observed in H/R upon CKMT2 overexpression as mitochondrial ROS (mtROS) production demonstrated a ≤26% decrease in mitochondrial superoxide production compared to MTS-GFP (**Fig. 2F**). Together, these results suggest that overexpression of CKMT2 improves cell viability by regulating ΔΨm and mtROS production independent of ATP production, which in turn allows for cardiac cell survival under H/R.

4. Phosphorylation of human CKMT2 occurs at various conserved sites

We then considered investigating the possible phosphorylation of CKMT2 by collecting protein phosphorylation data from Phosphosite.org. A cross-literature review and quantitative analysis of data from the site revealed the 3 phosphorylation sites with the most references: Tyr^{159} (Y159), Tyr^{255} (Y255), and Tyr^{368} (Y368) (**Table 1**, **Fig. 3A and B**). We then checked the conservation of these protein phosphorylation sites within protein domain families by collecting data from publicly available sources. If a site is highly conserved, it means that throughout its evolution the gene encoding the protein has served an important physiological purpose.[28] Of the phosphosites initially identified, tyrosine in phosphorylation sites 159 and 368 in CKMT2 were highly conserved across 150 diverse species, as revealed using ConSurf server.[25] Tyrosine in 255 was only conserved for less than 1% of the species scanned.

We then confirmed for the effect of CKMT2 phosphorylation sites during deletion or mutation by performing analysis on Y159A, Y255A, and Y368A, substituting tyrosine for alanine which is made up of beta carbon but devoid of side-chain chemistry. First, we investigated the effects of mutation on protein stability using STRUM prediction software.[29] Analysis of all 3 phosphomutants sited resulted in negative ΔΔG values, denoting that these mutations do not affect the native folded structure of the protein and rather affects posttranslational modifications specifically phosphorylation (**Table 2**). Using HOPE mutant analysis server which predicts susceptibility of mutant proteins to disease or deleterious effects,[26] we found that Y159 and Y368 were the most susceptible to deleterious effects during mutation (**Fig. 3C**) which indicate the importance of these phosphorylation sites.

Table 1. Number of HTP proteomic references in selected CKMT2 residues Y159, Y255, and Y368

Phosphosite	Human sequence
T151	DLDASKITQGQFDEH
Y159	QGQFDEHYVLSSRVR
Y207	KGDLAGRYYKLSEMT
Y208	GDLAGRYYKLSEMTE
S211	AGRYYKLSEMTEQDQ
Y255	ARGIWHNYDKTFLIW
Y313	MWNERLGYILTCPSN
S319	GYILTCPSNLGTGLR
S343	LSKDPRFSKILENLR
T361	RGTGGVDTAAVADVY
Y368	TAAVADVYDISNIDR
Y393	IVIDGVNYLVDCEKK

Only phosphorylated residues with HTP references more than 10 as indicated in Phosphosite.org were included in the table. Shaded boxes are the 3 with the most HTP references.
HTP, high throughput paper.

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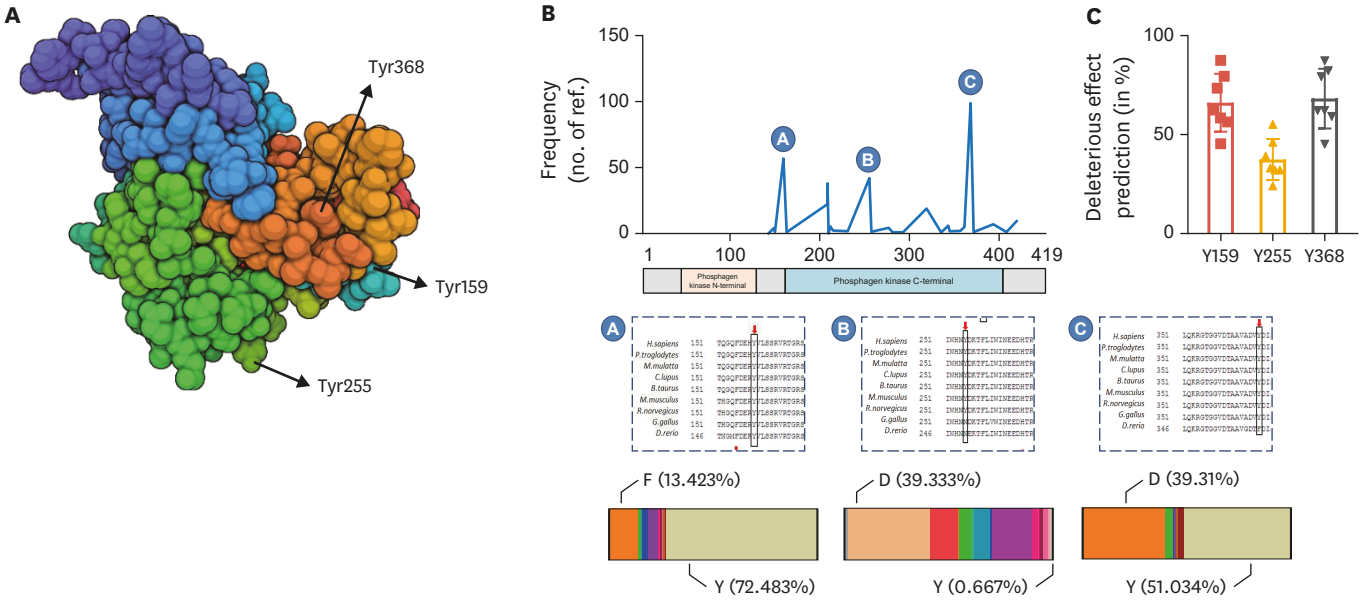

Fig. 3. Phosphorylation of human CKMT2 occurs at various conserved sites. (A) Cartoon representation of CKMT2 and (B) frequency of CKMT2 phosphorylation sites based on a cross-literature review of high throughput paper at Phsophosite.org, and quantitative analysis of residue conservation at the 3 most referenced CKMT2 phosphorylation sites as calculated by ConSurf. (C) Average percent confidence of 7 prediction servers regarding the deleterious effect of phosphomutations, as calculated by HOPE.

Table 2. Effect of site-directed mutagenesis in CKMT2 protein stability and native folding

Position	Wild-type	Mutant type	ΔΔG
159	Y	A	−1.13
255	Y	A	−2.19
368	Y	A	−1.97

Data are represented as below: Y, tyrosine; A, alanine; ΔΔG, the effects of these mutations to protein stability as measured by Gibbs' free energy values.
Y residues in Y159, Y255, and Y368 were changed to A. A more negative value reflects a more stable protein.

5. Genetic substitution of CKMT2 affects mitochondria function under H/R condition

Mitochondrial protein phosphorylation is an important regulator of mitochondrial functions and tissue ATP levels, and thus could be potential targets for therapeutic interventions to mitigate the effects of ischemic injury.[30] However, this possibility has not been demonstrated yet. Based on **Fig. 1D**, we found that CKMT2 could be post-translationally modified, we sought whether phosphorylation occurs at specific sites of the protein and how it can affect mitochondrial function. To address this, we constructed single amino acid substitution mutants of Y159A-GFP, Y255A-GFP, Y368A-GFP and investigated for mitochondria location, cell survival, ATP content, and mitochondria function using AC16 cells (**Fig. 4A**). Y159A-GFP, Y255A-GFP, and Y368A-GFP mutants were localized in the mitochondria; however, curved thread-like entanglements were observed in phosphomutants as opposed to the thread-like manner of CKMT2-GFP (**Fig. 4B**).

ATP production is directly linked to the CK/PCr shuttle and thus we, we then investigated whether the earlier decrease in ATP production could be linked to CK activity. We transfected empty pcDNA3.1(−) with various CKMT2 substitution mutants constructs in HEK293 cells and were purified using HIS-resin. CK activity was found to be significantly reduced in all CKMT2 substitution mutants compared to CKMT2 (compared to WT, Y159A: ≤80% decrease, Y255A: ≤45% decrease, Y368A: ≤77% decrease, **Fig. 4C**). These results suggest that CK

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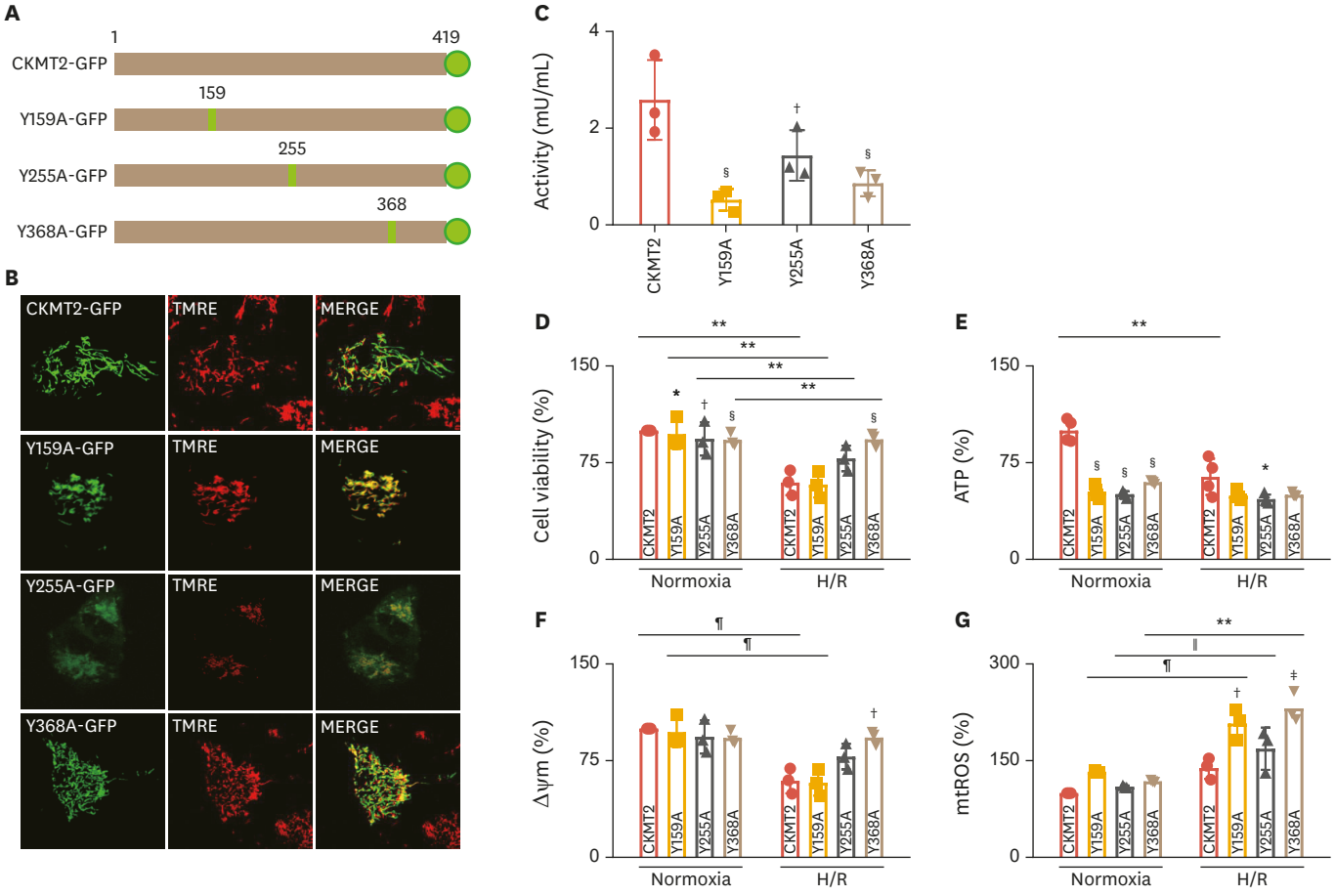

Fig. 4. Single mutation in CKMT2 phosphorylation sites reduces mitochondria function under H/R condition. (A) Schematic diagram of CKMT2 and CKMT2 alanine substitution mutants in Tyr^{159} (Y159A), Tyr^{255} (Y255A), and Tyr^{368} (Y368A). (B) CKMT2-GFP and CKMT2 substitution mutant-GFP were transfected into AC16 cells for 24 hours. Mitochondria were labeled with TMRE (red). Cells were visualized using confocal microscopy. (C) CKMT2 isoenzyme activity assays from the HIS-purified lysates of pcDNA6.1 or pcDNA6.1-CKMT2, and pcDNA6.1-CKMT2 substitution mutants. The horizontal axis shows the transgene and each graph corresponds to individual isoenzymes CKMT2/CKMT2 substitution mutants. (D, E) CKMT2-GFP and CKMT2 substitution mutant-GFP were transfected into AC16 cells for 24 hours. Transfected cells were incubated in 2% O_2 for 18 hours to simulate hypoxia condition, followed by 2 hours re-oxygenation at 18% O_2, before conducting cell viability and intracellular ATP content analysis. (F, G) CKMT2-GFP and CKMT2 substitution mutant-GFP were transfected into AC16 cells for 24 hours. After hypoxia/re-oxygenation, cells were stained with 200 nM TMRE and 1 μM Mito-SOX for 30 minutes and analyzed by flow cytometry. Data were normalized to MTS-GFP. The data are expressed as the mean±standard error of the mean of 3 or 4 independent experiments, where applicable. (C) Two-tailed unpaired Student's *t*-tests. (D-G) Two-way analysis of variance, *post hoc* Tukey's multiple comparisons test.
ATP, adenosine triphosphate; Δψm, mitochondrial membrane potential; mtROS, mitochondrial reactive oxygen species.
$^{*}p<0.01$, $^{\dagger}p<0.01$, $^{\ddagger}p<0.001$, $^{\S}p<0.0001$ for intra-subgroup comparison, $^{\|}p<0.01$, $^{\P}p<0.01$, $^{**}p<0.0001$ for inter-subgroup comparison.

inactivation due to dephosphorylation of CKMT2 affected CK activity, which is directly linked to ATP production.

Concurrently, the cell viability of single amino acid substitution mutant cells in normoxia was significantly lower by as much as ≤35% compared to CKMT2-GFP cells, and by as much as ≤62% during H/R (**Fig. 4D**). The decrease in cell viability of substitution mutant cells can be attributed to the pronounced decreased mitochondrial dysfunction, as shown by a significant decrease in ATP content and a significant decrease in Δψm and a significant increase in mtROS production during H/R (**Fig. 4E-G**). The change in levels of survival and ATP contents may be a result of the regulation of CKMT2. Taken together, these results indicating that CKMT2 inactivation due to dephosphorylation of CKMT2 may affect mitochondria function.

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6. CKMT2 mutation influence mitochondria protein expression

To determine whether substitution mutants of CKMT2 regulates mitochondrial proteins, western blotting was performed on transfected AC16 cells of CKMT2 and substitution mutants of CKMT2 under normoxia and H/R (**Fig. 5A**). Peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) is the main regulator of oxidative phosphorylation and fatty acid oxidation, its absence is known to result in contractile dysfunction and heart failure.[31] PGC-1α expression was significantly lower by ~20%–~50% in substitution mutants of CKMT2 under normoxia and decreased by as much as ≤70% during H/R. Transcription factor ERRα was also significantly lower in substitution mutants of CKMT2 under normoxia and H/R. Prohibitin 1 (PHB1), which is responsible for mitochondrial stabilization, was also lower in phosphomutations in normoxia and H/R (**Fig. 5B**). Taken together, these data suggest that CKMT2 can regulate mitochondrial protein expression.

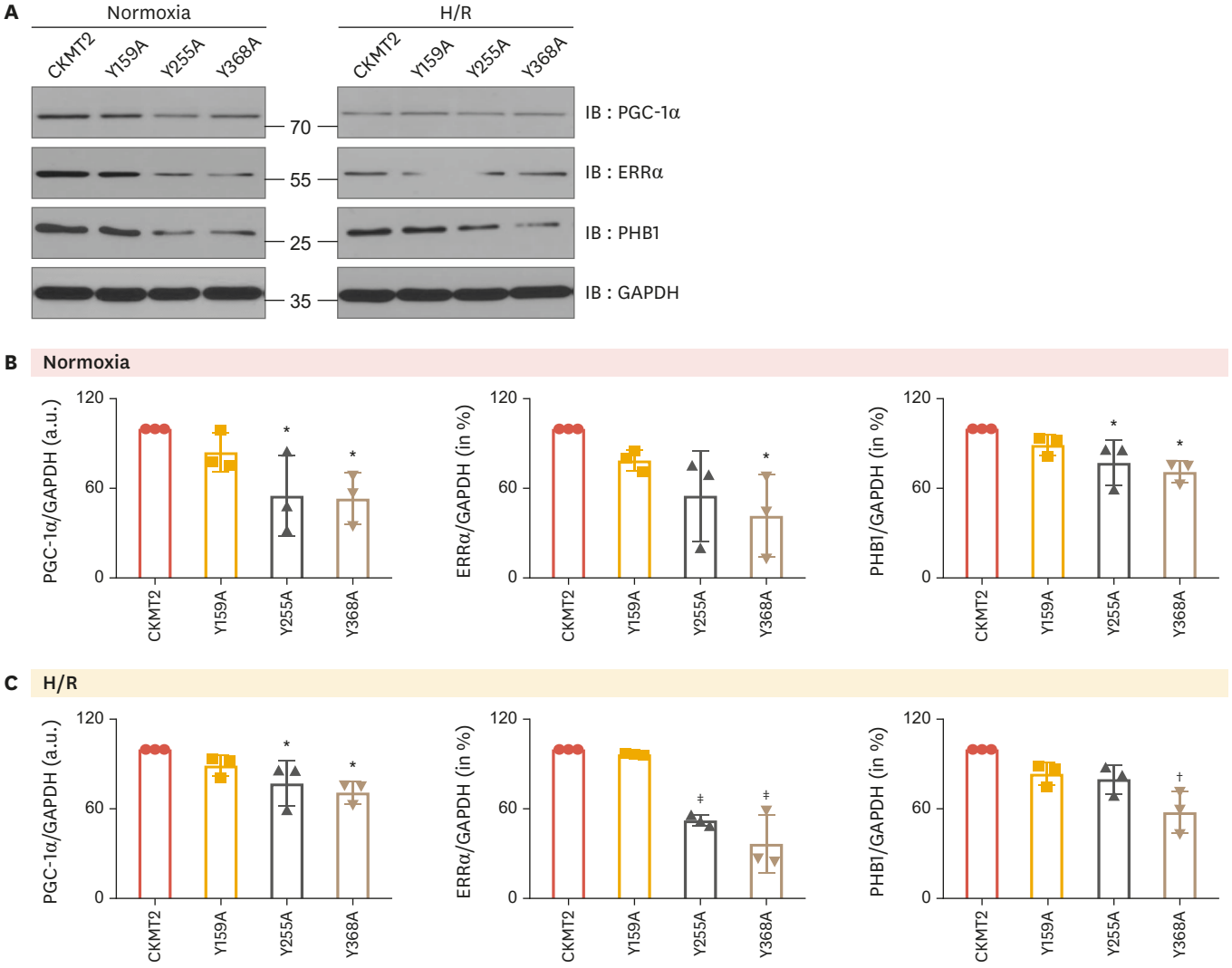

Fig. 5. CKMT2 influence mitochondria protein expression. (A) Representative figures and densitometric ratios of (B) PGC-1α, ERRα, and PHB1 during single substitution mutation under normoxic and H/R conditions. CKMT2-GFP and CKMT2 substitution mutant-GFP were transfected into AC16 cells for 24 hours. Ratios of proteins to GAPDH were densitometrically quantified using ImageJ. (B) One-way analysis of variance, *post hoc* Dunnett or Holm-Sidak multiple comparisons test. PGC-1α, proliferator-activated receptor γ coactivator-1α; ERRα, estrogen-related receptor-α; PHB1, prohibitin 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; H/R, hypoxia/reoxygenation.
*p<0.01, †p<0.01, ‡p<0.001.

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DISCUSSION

Here we report the regulatory role of CKMT2 overexpression in mitochondrial function which led to cardiac cell protection during H/R in an *in vitro* setting. We also determined and characterized 3 essential phosphorylation sites in CKMT2: Tyr^{159} (Y159), Tyr^{255} (Y255), and Tyr^{368} (Y368). Mutations in these functional phosphorylation sites render a significant decrease in CKMT2 activity and mitochondrial dysfunction, which affected the cardioprotective capability of CKMT2, especially during H/R.

Our results compound on previous data which demonstrate the cardioprotective effect of CKMT2 overexpression during H/R.[15,32] In our model, H/R induced a modest decrease in cell viability which was ameliorated by CKMT2 overexpression. On the other hand, ATP level remained unchanged during hypoxia and even during CKMT2 overexpression, similarly observed in *in vivo* CKMT2 overexpression models, which attributed possible elevated CKMT2 as a transformer of adenylate energy charge or cellular adenosine release under ischemic conditions.[27] Previous reports have also observed near-normal ATP levels until end-stage heart failure which is attributed to the buffering capacity of PCr and the CK equilibrium constant strongly favoring ATP synthesis.[33] In this case, the influence of CKMT2 overexpression may possibly directly affect the Δψm-ROS axis.

Δψm-ROS are highly dependent on each other, as ROS production depends exponentially on ΔΨm and a high ΔΨm stimulates the mitochondrial respiratory chain to significantly increase ROS production.[34] Thus, a balance between ΔΨm and ROS should be maintained to avoid excessive ROS production which can lead to various pathologies. ROS it in itself is also an attractive target to address these pathologies. It was previously reported that CK is the main target of ROS in heart myofibrils through the oxidation of sulfhydryl groups, and its deactivation results in a lower intramyofibrillar ATP-to-ADP ratio.[35] Later on, CKMT2 activity was discovered to play an antioxidant role against oxidative stress albeit in the brain instead of a heart, thereby reducing mitochondrial ROS generation through an ADP-recycling mechanism.[36] Moreover, CKMT2 is hypersensitive to inactivation by ROS and other ischemia by-products (i.e., peroxynitrite).[37]

We further evaluated the cardioprotective role of CKMT2 by performing mutation on conserved phosphorylation sites. The current study is the first to characterize the functional role of Y159, Y255, and Y368 in CKMT2 through site-directed mutagenesis. The substitution of a single amino acid in a protein sequence can significantly alter a protein's stability (ΔΔG), where a positive ΔΔG represents a destabilizing mutation and a negative ΔΔG represents a stabilizing mutation.[38] Because the mutations introduced in CKMT2 did not affect the native folding and stability of the protein, there could have been negligible effect to ligand binding, allosteric coupling, catalytic activity. Although our results demonstrated that CKMT2 was phosphorylated during perfusion and IPC in an animal model, human mRNA data and GEO data in disease models indicated that the mRNA expression was unchanged. We infer that the phosphorylation of CKMT2 is mediated by post-transcription regulation and the mutations introduced directly affected posttranslational modifications specifically phosphorylation which signals subsequent molecular mechanisms to proceed.[39]

Our current study demonstrates Y159, Y255, and Y368 as essential phosphorylation sites intersecting the Cr/PCr and ADP/ATP cycles which allows for the continuous production of energy supply and cell survival. Thus, activation of CKMT2 may be a normal event in cardiac

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cells. In a different study done in breast cancer cells, researchers found that activation of Y153 phosphorylation, through the HER2/ABL axis, stabilizes the other mitochondrial CK isoenzyme CKMT1 to increase the phosphocreatine energy shuttle and promote proliferation.[40] However, unlike the aforementioned study, we have not yet investigated potential mechanisms on Y159, Y255, and/or Y368 phosphorylation activation which may contribute to CK/PCr shuttle and ATP production in the cardiac setting and will merit future experiments. It will be interesting to find a tyrosine kinase that can phosphorylate the tyrosine residues of CKMT2, which in turn can regulate downstream mitochondria-related proteins.

Moreover, the dysregulation caused by the mutation and hypoxia can affect the PGC-1/ERR axis, which regulates synchronized shifts in ATP production, energy metabolism, and mitochondrial function in the heart. This dysregulation eventually results in a progressive deactivation of the PGC-1/ERR axis and a further reduction in ATP levels as it directly impacts ATP-coupled respiration and efficacy of electron transport chain (ETC).[41,42] Excessive amounts of mtROS are also produced as an unwanted by-product of a dysfunctional ETC, which is considered as its main source.[43] Since mtROS are mainly sourced from ETC, and PHB1 directly regulates ETC activity, PHB1 also has control in mtROS production, and in effect $\Delta\Psi m$ as well.[44] Also, PHB1 compounded on the observed mitochondrial dysfunction as it also modulates mitochondrial integrity and bioenergetics under oxidative stress and is a known regulator of mitochondrial fragmentation, which can explain the thread-like fragmentation observed in the mitochondria of phosphomutants.[45]

In conclusion, here we report the regulatory role of CKMT2 overexpression in mitochondrial function which led to cardiac cell protection during H/R in an *in vitro* setting. We also determined and characterized 3 essential phosphorylation sites in CKMT2: Tyr^{159} (Y159), Tyr^{255} (Y255), and Tyr^{368} (Y368). Mutations in these functional phosphorylation sites render a significant decrease in CKMT2 activity and mitochondrial dysfunction, which affected the cardioprotective capability of CKMT2, especially during H/R. Our current study demonstrates Y159, Y255, and Y368 as essential phosphorylation sites intersecting the Cr/PCr and ADP/ATP cycles which allows for the continuous production of energy supply and cell survival (**Fig. 6**). Our current study offers a different perspective on cardioprotection during I/R and H/R injury by screening potential offering a novel target in rather than the conventional method of targeting infarct size reduction. However, we have yet to fully validate the phosphorylation state of these sites in future experiments using a phospho-antibody specific to the site. Moreover, we also have to identify a tyrosine kinase that can phosphorylate the tyrosine residues of CKMT2, which in turn can regulate downstream mitochondria-related proteins and can be used as a target for future therapeutic approaches to cardiac diseases.

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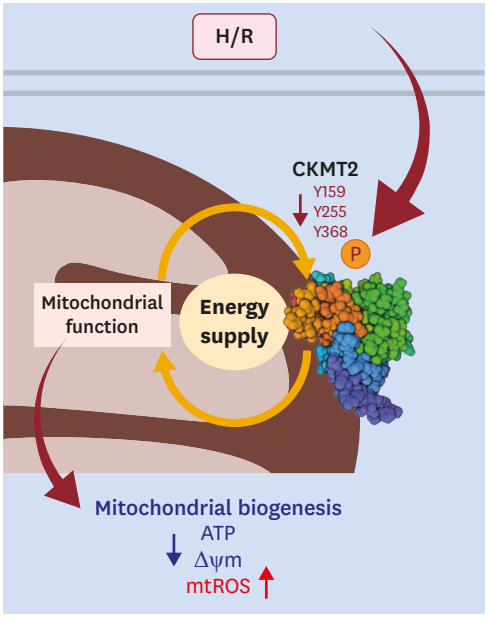

Fig. 6. Phosphorylation of CKMT2 in Y159, Y255, and Y368 confer cardioprotection against H/R injury in AC16 cells. Schematic diagram indicating the specific tyrosine residues affected during H/R, which led to the regulation of creatine kinase activity and mitochondrial function. Created with BioRender.com.
H/R, hypoxia/reoxygenation; ATP, adenosine triphosphate; Δψm, mitochondrial membrane potential; mtROS, mitochondrial reactive oxygen species.

SUPPLEMENTARY MATERIALS

Supplementary Data 1

Full enriched biological processes, molecular function, and cellular component terms in control ischemic-preconditioned rat hearts using Database for Annotation, Visualization and Integrated Discovery (https://david.ncifcrf.gov).

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Supplementary Table 1

List of phosphorylated cardiac mitochondria-specific proteins during perfusion, ischemia only, I/R, and IPC

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Supplementary Fig. 1

(A) Gene Ontology enriched terms of molecular function and (B) protein class were analyzed using Protein Analysis Through Evolutionary Relationships (http://www.pantherdb.org/about.jsp). Top 10 enriched (C) biological processes, (D) molecular function, and (E) cellular component terms in ischemic-preconditioned rat hearts using Database for Annotation, Visualization and Integrated Discovery (https://david.ncifcrf.gov). Full list of terms can be viewed in Supplementary Data 1 provided.

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Supplementary Fig. 2

(A) Increased and (B) decreased protein functional interaction networks in heart phosphoproteome in control and ischemic preconditioned comparison groups. Interactions were mapped using Search Tool for the Retrieval of Interacting Genes/Proteins database version 11.0 with a confidence cutoff of 0.4.

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