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ORIGINAL RESEARCH

Cardioprotection Through Pharmacological Activation of Sirtuin 5 in a Murine Model of Acute Myocardial Infarction

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Purpose: Sirtuins (SIRTs) play a critical role in redox and metabolic regulation of the myocardium; however, the cardioprotective potential of SIRT5 in terms of infarct size (IS) reduction is still elusive. Herein, we employed the newly synthesized SIRT5-specific agonist, MC3215, developed by our group, to explore for the first time the pharmacological activation of SIRT5 as a target for cardioprotection.

Methods and Results: In in vitro screening experiments, SIRT1 and SIRT5 agonists, namely, MC2606 and MC3215, at 1–20 μM were added to cardiomyoblasts (H9c2) and human endothelial cells (EA.hy-926) during 24 h hypoxia/2 h reoxygenation (H/R). SIRT1 and SIRT5 agonists mitigated H/R injury. Male C57BL/6J mice underwent 30 min ischemia (I) followed by 2 h or 24 h reperfusion (R). Mice received vehicle, the SIRT1 or SIRT5 agonists at 20 and 30 mg/kg at the 20th min of ischemia, and IS was quantified via triphenyl-tetrazolium chloride staining (n=5–7/group). MC3215-mediated SIRT5 activation reduced IS at 24 h R at 20mg/kg compared to controls (25.18±2.7% vs 38.80±4.7%). MC3215 treatment resulted in reduced protein malonylation in all experimental settings. Targeted mass-spectrometry-based metabolomics in the ischemic heart at the 10th min of R suggested increased fatty acid oxidation, as indicated by increased N^3-Trimethyllysine and D-pantothenate. Concomitantly, molecular analysis indicated that the SIRT5 agonist activated AMPKα and Reperfusion Injury Salvage Kinase (RISK) pathway. Additionally, at 3 h reperfusion, MC3215 led to increased mitofusin 2 without altering apoptosis, paving towards improved mitochondrial dynamics. Co-administration of SIRT5 inhibitor, TW-37, abrogated MC3215-mediated cardioprotection.

Conclusion: SIRT5 pharmacological agonism emerges as a novel cardioprotective target, leading to RISK pathway activation and mitochondria-related metabolic effects, converging at salvaging ischemic myocardium from I/R injury.

Keywords: sirtuins, SIRT5, myocardial ischemia/reperfusion injury, cardioprotection

Introduction

Myocardial infarction (MI) is a leading cause of morbidity and mortality worldwide, resulting in cardiac damage due to insufficient energy and oxygen supply to the myocardium.[1] Timely restoration of coronary flow is essential to reduce infarct size (IS), but paradoxically, reperfusion exacerbates cardiac injury, a phenomenon referred to as myocardial ischemia/reperfusion injury (IRI).[2] Cardioprotection in terms of IS reduction is needed in addition to rapid reperfusion

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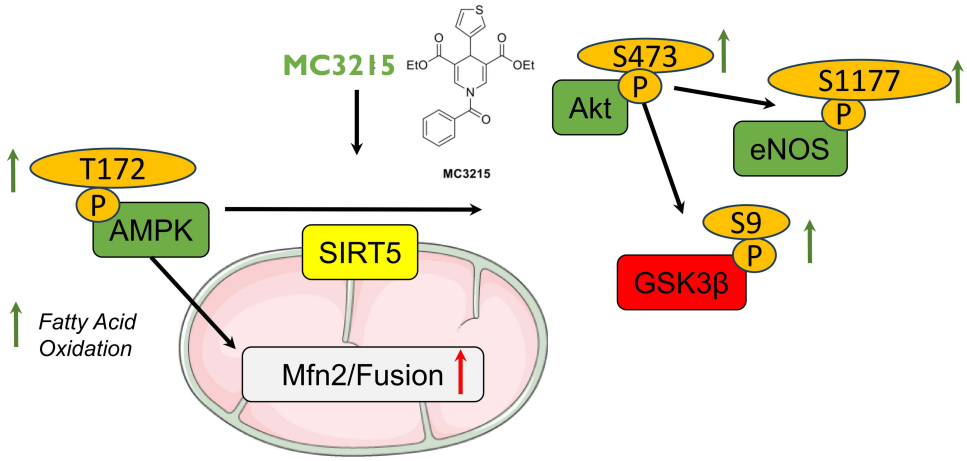
Graphical Abstract



therapy, but translation of pharmacological and mechanical interventions into clinical practice has proven to be challenging, underscoring the complexity of IRI mechanisms.[3] Current approaches focus on optimizing the timing and dosage of interventions and continuing the development of novel cardioprotective targets and strategies.

Sirtuins (SIRTs) are NAD^+-dependent enzymes highly conserved across species, which catalyze the reversible removal of acetyl groups from both histone tails and non-histone proteins. Importantly, their impact on cellular homeostasis has aroused growing scientific interest.[4] SIRT1 is a well-recognized target for cardioprotection. SIRT1 has bestowed cytoprotection in cardiomyocytes after hypoxia-reoxygenation by upregulating antioxidant molecules[5] and by promoting autophagy while reducing hypoxia-induced cardiotoxicity both in vitro and in vivo.[6] Cardiac-specific SIRT1 knockout increases oxidative stress, inflammation, IS, and apoptosis.[5] The levels of SIRT1 activation are critical to its protective effects; excessive cardiac SIRT1 expression can lead to mitochondrial dysfunction and increased oxidative stress.[7] Recently, SIRT5 has gained attention in the cardiovascular system.[8] It suppresses oxidative-stress-induced apoptosis, playing a significant role in the cardiac stress response,[9] and it contributes to increased oxygen utilization by the ischemic heart, thereby improving cardiac dysfunction during IRI.[10] Moreover, studies have demonstrated that SIRT5 knockout negatively affects the heart's capacity to recover from ischemic injury.[11] However, the cardioprotective potential of SIRT5 remains elusive since it is primarily based on studies using genetic manipulation,[11] and to the best of our knowledge, SIRT5 pharmacological activation has not yet been investigated as a putative cardioprotective strategy.

Our group has previously described the scaffold optimization, the synthesis, and the pharmacological evaluation in terms of SIRT-isoform specificity of several SIRT agonists.[8,12–24] Two of our recently described sirtuin agonists, namely, MC2606 and MC3215 (Supplementary Figure 1), were chosen based on their in vitro specificity on activating SIRT1 and SIRT5, respectively, using mass Spectrometry (MS) in a coupled enzymatic assay.[13] Therefore, the primary aim of this study was to investigate the cardioprotective potential of the specific SIRT5 agonist, MC3215, against IRI, providing valuable and novel insights into the role of SIRT5 in IRI. MC2606, the SIRT1 agonist was used, taken the proven cardioprotective potential of SIRT1.[6] First, we aimed to screen the relevant cytoprotective dosage of MC3215 and MC2606 in cardiomyoblasts (H9c2 cells) and human endothelial cells (EA.hy-926) in an in vitro model of IRI. In the same model, we aimed to assess the cytoprotective potential in comparison to SRT1720, a reference compound for SIRT1 activation previously described to alleviate IRI.[25] Next, we aimed to examine the cardioprotective potential of the SIRT5 agonist in vivo in a translational murine model of IRI. Additionally, we sought to identify the underlying cardioprotective mechanism of the SIRT5 agonist, focusing on metabolism and endogenous cardioprotective mechanisms. Since it has

been shown that SIRT5 may regulate mitochondrial dynamics,[26,27] we aimed to investigate its impact on mitochondrial regulators of apoptosis, fusion, and fission. Finally, we examined the target engagement of MC3215 to SIRT5 in vivo and determined the potential binding geometry for the novel SIRT5 activator, MC3215, using a structure-based in silico approach.

Materials and Methods

An extensive version of Materials and Methods is provided in the Supplementary material.

Pharmacological Agents

SRT1720, N-(2-(3-(piperazin-1-ylmethyl)imidazo[2,1-b]thiazol-6-yl)phenyl)quinoxaline-2-carboxamide, a previously described SIRT1 agonist,[28] was used in vitro as a reference SIRT1 activating compound. TW-37, N-[4-[[2-(1,1-Dimethylethyl)phenyl]sulfonyl]phenyl]-2,3,4-trihydroxy-5-[[2-(1-methylethyl)phenyl]methyl] benzamide was used in vivo as an SIRT5 inhibitor.[29] MC2606 and MC3215 have been prepared according to the literature (Supplementary Figure 1).[13,19]

Experimental Protocol in vitro

The embryonic rat heart-derived cell line H9c2 and EA.hy-926 endothelial cells were obtained from ATCC (via Life Science Chemilab, Athens, Greece) and were cultured and subjected to hypoxia for 24 h and reoxygenation for 2 h (H/R). Cells were incubated with the SIRT5 agonist, MC3215, at three different concentrations: 1 μM, 10 μM, and 20 μM. SRT1720, an established SIRT1 agonist,[25] and MC2606,[13] a newly discovered SIRT1 agonist, were used to compare the cytoprotective effects of the different SIRT isoform activation. Cell viability and Western blot analysis were performed as described in the Supplementary material.

Experimental Protocols in vivo

Ethical Statement

A total of one hundred and thirty-three (133) male C57Bl/6J (13–14 weeks of age) mice were used according to ARRIVE guidelines.[30,31] Mice were bred and housed in the Animal Facility of the Biomedical Research Foundation Academy of Athens. All animal procedures were performed in compliance with the Presidential Decree 56/2013 for the protection of the animals used for scientific purposes, in harmonization to the European Directive 2010/63, and the experimental protocols were approved by the competent Veterinary Service of the Prefecture of Athens (Protocol number: 1388007/13/11/2023). Mice were housed in a maximum of 6 animals per cage in a specific pathogen-free facility under a 12-h light/dark cycle and received regular laboratory animal diet ad libitum. Surgical procedures and interventions were performed in compliance with the guidelines "Practical guidelines for rigor and reproducibility in preclinical and clinical studies on cardioprotection".[32,33] Randomization was performed for all the series of experiments, and the surgeries in mice and the IS calculations were performed by blinded investigators.

Investigation of SIRT5 Activation as a Cardioprotective Target in a Murine Model of Myocardial Ischemia Reperfusion

The experimental protocols are illustrated in Supplementary Figure 2. SIRT1 and SIRT5 agonists, MC2606 and MC3215, were tested in vivo in the setting of myocardial ischemia (I) and reperfusion (R). The initial in vivo dose (30 mg/Kg) for the sirtuin agonists (MC2606 and MC3215) was chosen based on a previous study[25] using SRT1720 in the same model of IRI. Mice (n=21) were randomized into 3 groups (n=7 per group): Control: treated with vehicle (5% DMSO); MC2606 at 30 mg/Kg; MC3215 at 30 mg/Kg (Supplementary Figure 2A). Mice underwent 30 min I/2h R, while compounds were administered intraperitoneally at the 20th min of ischemia. The timepoint of SIRT agonist administration was translationally selected according to clinical practice, since cardioprotective interventions in MI-patients are performed prior to the conduction of PCI. At the end of reperfusion, IS was determined as described in detail in the Supplementary material.

In a second series of experiments, we aimed to explore the cardioprotective effect with prolonged reperfusion, and 27 mice were randomized into the same three groups (n=9 per group) and subjected to 30 min I/24h R. At the end of reperfusion, IS was assessed (Supplementary Figure 2B).

In the third series of experiments, we aimed to optimize the dose of SIRT agonists, and 23 mice were randomized into 3 groups (n=7–8 per group) treated as follows: Control: treated with vehicle; MC2606 at 20 mg/Kg; MC3215 at 20 mg/Kg. Mice underwent 30 min I/24h R, and IS was determined.

To investigate the mechanism of SIRT5-mediated cardioprotection, we further employed a fourth and a fifth series of experiments with 10 min and 3 h reperfusion, respectively, as described in the Supplementary material (Supplementary Figure 2C).

Investigation of MC3215 Target Engagement in the Murine Model of Ischemia Reperfusion

Finally, to confirm the target engagement of MC3215 for SIRT5, we used a SIRT5 inhibitor (TW-37, 10 mg/Kg), in accordance with a previous in vivo study.[34] Mice (n=30) were randomized into 4 groups and treated as follows: Control (n=7): treated with 5% DMSO; MC3215 (n=7) at 20 mg/Kg; TW-37 (n=9) at 10 mg/Kg; TW-37 (10 mg/Kg) + MC3215 (20 mg/Kg) (n=7). Mice underwent 30 min I/2h R, and the compounds were administered intraperitoneally (TW-37 and DMSO or MC3215 were administered at the 5th and the 20th min of ischemia, respectively). At the end of reperfusion, IS was determined (Supplementary Figure 2D).

Statistical Analysis

All statistical analyses and graph preparation were performed using GraphPad Prism 8.5 analysis software (GraphPad Software, Inc., La Jolla, CA, USA). All the results were presented in graphs as mean ± standard deviation (SD). For comparisons between two groups, an unpaired, two-tailed Student's *t*-test was implemented, while for comparisons among three or more groups. One-way analysis of variance (ANOVA) with Tukey post hoc test for multiple pairwise comparisons was used. P values <0.05 were considered statistically significant and were reported accordingly.

Results

SIRT5 Activation Protects Against Hypoxia/Reoxygenation Injury in vitro

SIRT5 agonist, MC3215, and the SIRT1 agonist, MC2606, significantly restored the cell viability of H9c2 and EA.hy-926 cells exposed to H/R, at 1μM and did not alter cell viability in normoxia (Figure 1A and B). SRT1720 resulted in significant cytotoxicity at 10 μM and 20 μM in both cell lines and under both conditions (normoxia and hypoxia) (Supplementary Figure 3A-D). Only MC3215 resulted in an increased viability of H9c2 cells at 10 μM, while both MC2606 and MC3215 reduced the cell viability of EA.hy-926 cells at 20 μM. (Supplementary Figure 3A-D).

Among sirtuins, only SIRT5 exerts demalonylation potential,[35] and therefore, to verify that the activation of SIRT5 is related to MC3215-mediated protection against H/R injury, we assessed lysine's malonylation status on both cell lines. SIRT5 has been shown to regulate protein malonylation both in the cytosol and mitochondria,[35] and therefore, we sought to investigate protein malonylation status in our in vitro experimental setting. MC3215 significantly decreased lysine malonylation in H9c2 lysates, cytosol, and mitochondria (Figure 1C and D). Similarly, lysine malonylation was decreased in EA.hy-926 endothelial cell lysates and cytosols, while malonylation in the mitochondrial fraction was marginally reduced (Figure 1E and F).

SIRT5 Activation Reduces Infarct Size in vivo

MC2606 and MC3215 were administered in vivo at 30 mg/Kg at the 20th min of ischemia, followed by 2 h of reperfusion. MC3215 significantly reduced IS compared to the vehicle-treated group (24,7 ± 6,3% for MC3215 vs 34,9 ± 4,7% for vehicle) (Figure 2A and B). Administration of the SIRT1 agonist MC2606 reduced IS (27.6 ± 5.7%, P=0.06 vs vehicle), without reaching statistical significance. The area at risk was similar among the groups, indicating a similar extent in ischemic insult. The treatments were well tolerated by mice (Figure 2C). Given that different biological pathways are activated during prolonged reperfusion,[36] the cardioprotective potential of SIRT5 and SIRT1 agonists was further examined at 24 h R. At the same dose, the compounds showed signs of toxicity, resulting in a high mortality rate of the mice at the first hours of reperfusion (Figure 2F). In the surviving mice, the data indicate that the

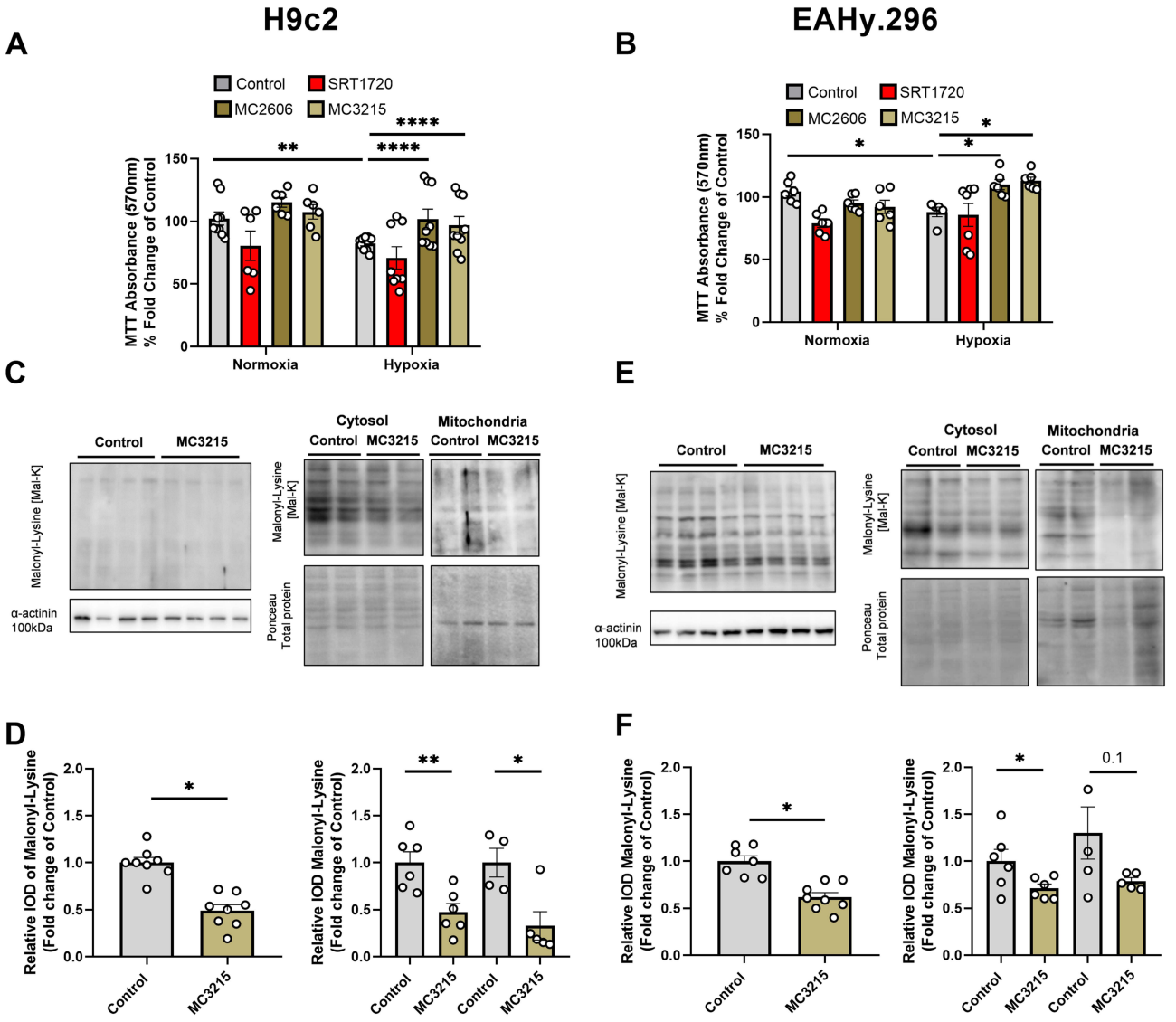


Figure 1 SIRT5 activation is protective in vitro against hypoxia/reoxygenation injury. % Viability in normoxia and hypoxia assessed by MTT conversion expressed as % fold change of normoxia after treatment with SIRT5 and SIRT1 agonists. Bar graphs of cell viability for (**A**) H9c2 and (**B**) EA.hy-926 endothelial cells, respectively, incubated with DMSO as vehicle (control) or with the indicated compounds at 1 μM. One-way ANOVA with Tukey's post hoc was performed for cell viability (*P<0.05, **P<0.01, ****P<0.0001). (**C**) Representative Western Blot images of malonyl-lysine (Mal-K) for H9c2 cell lysate, cytosol and mitochondria and (**D**) relative densitometry analysis of Mal-K normalized to α-actinin, normalized or total protein (Ponceau). Unpaired *t*-test, *P<0.05, **P<0.01. (**E**) Representative Western Blot images of Mal-K for EA.hy-296 endothelial cell lysate, cytosol and mitochondria and (**F**) relative densitometry analysis of Mal-K normalized to α-actinin, normalized or total protein (Ponceau). Unpaired *t*-test, *P<0.05, **P<0.01.

SIRT5 activator, MC3215, decreased IS, and that the area at risk was similar among the groups (Figure 2D and E). Consequently, the dose was reduced to 20 mg/Kg, and the experiments were repeated at 24 h R. The mice tolerated the treatment well without any significant signs of toxicity and mortality (Figure 2I). Only the SIRT5 agonist, MC3215, exerted cardioprotective potential at 20 mg/kg (25,2 ± 2.7% for MC3215 vs 38,8±4,7% for vehicle) (Figure 2G and H). The potential organ toxicity of MC3215 was examined at 20mg/kg. MC3215 administration did not cause significant changes in liver and kidney parameters, determined in plasma, compared to the control group (5% DMSO) (Supplementary Table 1). Collectively, the SIRT5 agonist led to a greater reduction in IS compared to the SIRT1 activator, and therefore, molecular mechanisms related to SIRT5 activation were further explored.

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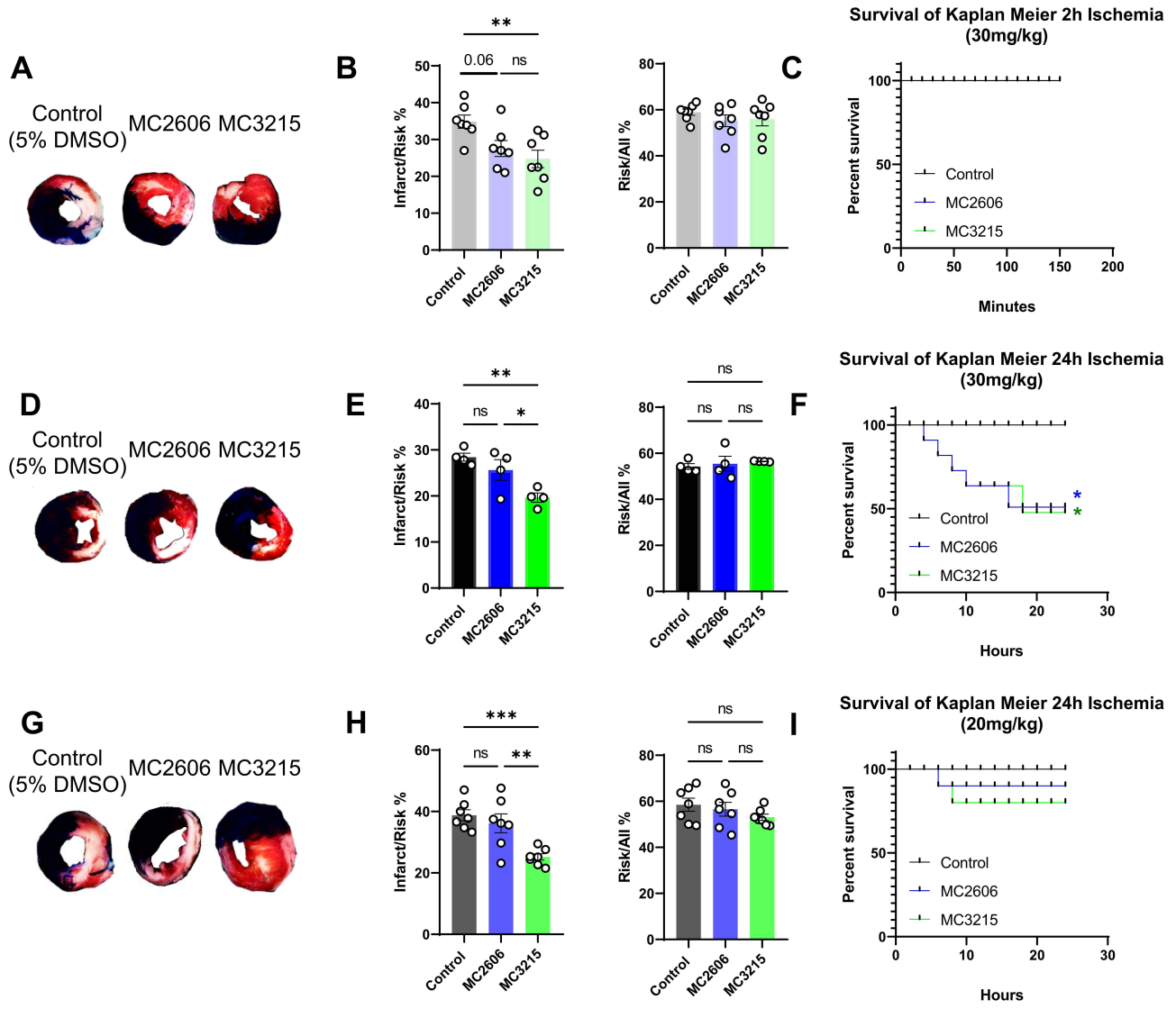

Figure 2 MC3215 reduces infarct size in the murine model of ischemia reperfusion injury. Representative images of myocardial infarcts are depicted (blue/dark is due to Evans Blue staining; red and white account for the area at risk; white area is denoted as the infarcted tissue). (**A**) Evans's blue/TCC double-stained heart slices after 30 min ischemia, 2 h of reperfusion of mice treated with vehicle or the SIRT agonists at 30 mg/Kg. (**B**) The effects of SIRT agonists at 30 mg/Kg on infarct size are expressed as a percentage of the area at risk (% Infarct/Risk), and the area at risk is expressed as a percentage of the total left ventricle area (% Risk/All) (n=7 per group, mean ± SD, **P< 0.01 vs the Control group). (**C**) Representative graph of Kaplan Meier's survival after 30min ischemia and 2h of reperfusion at 30 mg/Kg. (**D**) Evans's blue/TCC double-stained heart slices after 30 min ischemia and 24 h reperfusion of mice treated with vehicle or the SIRT agonists at 30 mg/Kg. (**E**) Bar plots with % Infarct/Risk and % Risk/All determined upon 30 min ischemia and 24 h reperfusion, n=4 per group, mean ± SD. (**F**) Representative graph of Kaplan Meier's survival after 30 min ischemia and 24h reperfusion at 30 mg/Kg. (**G**) Evans's blue/TCC double-stained heart slices after 30min ischemia and 24 h reperfusion of mice treated with vehicle or the SIRT agonists at 20 mg/Kg. (**H**) Bar plots with % Infarct/Risk and % Risk/All determined upon 30 min ischemia and 24h reperfusion at 20mg/kg, n=7 per group, mean ± SD, ***P< 0.001 vs the Control group. (**I**) Representative graph of Kaplan Meier's survival after 30min I/24h R at 20 mg/Kg. One-way ANOVA with Tukey's post hoc was performed for IS assessment.

SIRT5 Activation Decreases Myocardial Lysine Malonylation in vivo and Activates Cardioprotective Signaling

SIRT5, besides being the only sirtuin inducing demalonylation, is also a protein lysine desuccinylase. Since the role of protein malonylation and succinylation is still under investigation in IRI, these modifications were examined upon 30 min I/10 min R. We found that lysine malonylation and succinylation are not altered at early reperfusion compared to sham-operated mice (Figure 3A and B). Administration of MC3215 led to a significant reduction in the malonylated lysine at the 10th min of R compared to the control group in the ischemic myocardium, confirming our in vitro findings and verifying

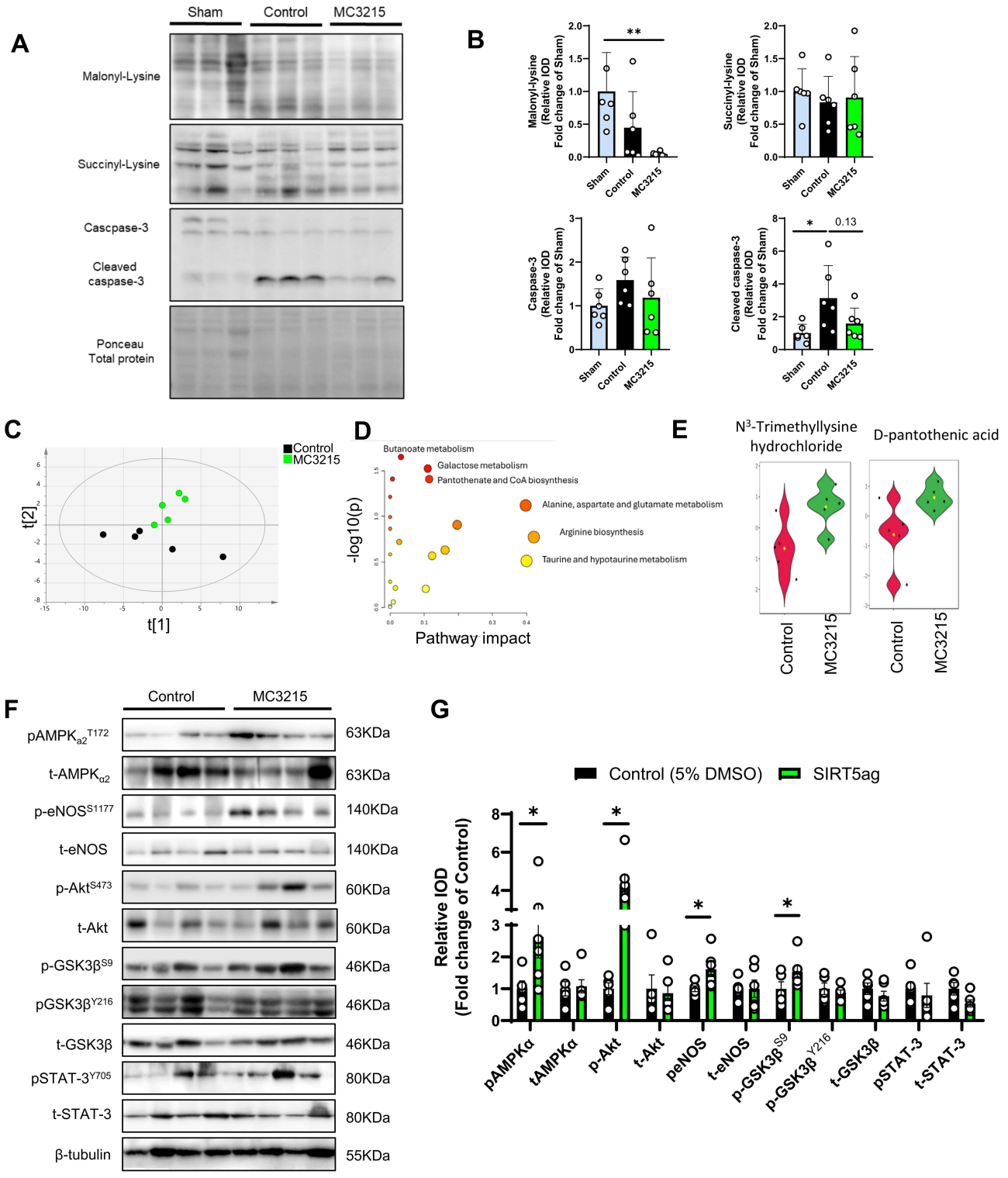

Figure 3 MC3215 increases SIRT5 activity in vivo and induces cardioprotection through activation of AMPKα and RISK pathway. (**A**) Representative Western Blot images and (**B**) relative densitometry analysis at the 10th min of reperfusion of malonyl-lysine, succinyl-lysine and caspase-3normalized to Total Ponceau. (**C**) Three-dimensional representation of principal components analysis (PCA) of the targeted metabolomic analysis of the heart (n=5 per group). (**D**) Metabolite enrichment overview of the top 25 metabolic pathways and (**E**) violin plot of the statistically significant metabolites emerged from the metabolomic analysis and MetaboAnalyst 5.0 software with enrichment and *P* value ascension. (**F**) Representative Western Blot images and (**G**) relative densitometry graphs at the 10th min of reperfusion of (phospho)-AMPKα (T172), (phospho)-eNOS (S1177), (phospho)-Akt (S473), (phospho)-GSK3β (S9 and Y216), and (phospho)-STAT-3 (Y705). All proteins were run in the same SDS-PAGE gel and shared the same β-tubulin as a loading control. Western blot data were analyzed with an unpaired *t*-test (*P< 0.05, **P< 0.01).

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SIRT5 activation in vivo. MC3215 did not lead to significant differences in protein succinylation (Figure 3A and B). Then, we sought to understand the mechanism of myocardial cell death alleviation by MC3215, and we examined apoptosis, focusing on caspase-3 and cleaved caspase-3 levels. At the 10th min of R, full-length caspase-3 did not differ among groups, and cleaved caspase-3 was significantly increased in the Control group vs Sham-operated animals (Figure 3A and B). However, MC3215 marginally reduced cleaved caspase-3 levels, suggesting that the mechanism of cardioprotection afforded by MC3215 and SIRT5 agonism is not tightly related to apoptosis at this timepoint.

Since we know that SIRT5 activation may promote metabolic flexibility, enabling cells to switch between different energy substrates based on availability and demand,[10] we first focused on metabolism. Reversed-phase liquid chromatography coupled to a QTOF mass spectrometry (MS) analyzer was employed for the metabolomics analysis of heart and plasma samples. The identified metabolites were used for multivariate comparisons. The principal component analysis (PCA) models successfully separated the heart and plasma samples upon treatment with SIRT5 agonist from the control group (Figure 3C and Supplementary Figure 4A-C). Pathway enrichment analysis of the metabolites in the heart revealed "Alanine, aspartate, and glutamate metabolism" and "Pantothenate and CoA biosynthesis" as differentially regulated by SIRT5 activation (Figure 3D). In the ischemic heart, MC3215 administration significantly increased N^3-trimethyllysine hydrochloride, a metabolic intermediate for carnitine biosynthesis and essential for the fatty acid transport in mitochondria,[37] and D-pantothenic acid, a metabolite participating in coenzyme A formation and fatty acid oxidation (FAO)[38] (Figure 3E).

We further investigated the cardioprotective signaling at the 10th minute of R. The MC3215 treatment activated adenosine monophosphate-activated protein kinase a (AMPKα), which coordinates anabolic and catabolic processes within the cell, including FAO, without altering its expression (Figure 3F and G). AMPK has been previously linked to protein kinase B (Akt) and the inhibition of glycogen synthase kinase-3 beta (GSK3β),[39] which is also an output for the cardioprotective signaling of the reperfusion injury salvage kinase (RISK) pathway.[36] We assessed the phosphorylation of Akt, endothelial nitric oxide synthase (eNOS), and GSK3β at the same time-point.[40] MC3215 treatment activated Akt and eNOS, while deactivating GSK3β by phosphorylation, respectively (Figure 3F and G). Moreover, we examined the activation of the signal transducer and activator of transcription 3 (STAT3), which is causally associated with cardioprotection[41] and has been identified as a SIRT5 interactor.[10] In our experimental setting, SIRT5 agonist MC3215 did not alter the phosphorylation and expression of STAT-3 (Figure 3F and G), and therefore its cardioprotective potential was linked to the activation of the AMPK-Akt-GSK3β axis.

Finally, dihydroethidium (DHE) staining and malondialdehyde (MDA) assay were performed, as markers of superoxide anion and lipid peroxidation, respectively. No significant difference between control and MC3215-treated mice regarding the intracellular ROS and MDA accumulation was detected (Supplementary Figure 5A-C), suggesting that MC3215 does not influence superoxide anion production and lipid peroxidation at this particular timepoint. Collectively, our results demonstrate that the SIRT5 agonist mediates the activation of cellular metabolism and cardioprotective pathways.

SIRT5 Activation Contributes to Increased Mitofusin 2

Several mechanisms contributing to myocardial damage and dysfunction occur at later stages of reperfusion, including alterations in mitochondrial dynamics.[42] Therefore, we expanded our investigation for the effect of MC3215 on myocardial signaling at 3 h of R, and we performed cellular fractionation, isolating the cytosolic and mitochondrial fractions from the ischemic myocardium of the mice. At 3 h of reperfusion, we observed a significant reduction in mitochondrial content in the infarcted mice in comparison to Sham-operated animals, which was not rescued by the SIRT5 agonist (Figure 4A).

Given that SIRT5 regulates protein malonylation in both the cytosol and mitochondria,[35] we investigated lysine malonylation in the respective subcellular fractions. We found that at 3 h of R, protein malonylation of the Control group was marginally increased compared to Sham in the cytosolic fraction. MC3215 treatment decreased lysine malonylation in the cytosolic proteins at the same time-point, confirming pharmacological activation of SIRT5 (Figure 4B and C). Malonylation of proteins in the mitochondrial fraction was similar among the groups (Figure 4B and C), indicating that the SIRT5 agonist alters this protein modification mainly in the cytosol. Regarding lysine succinylation of cytosolic

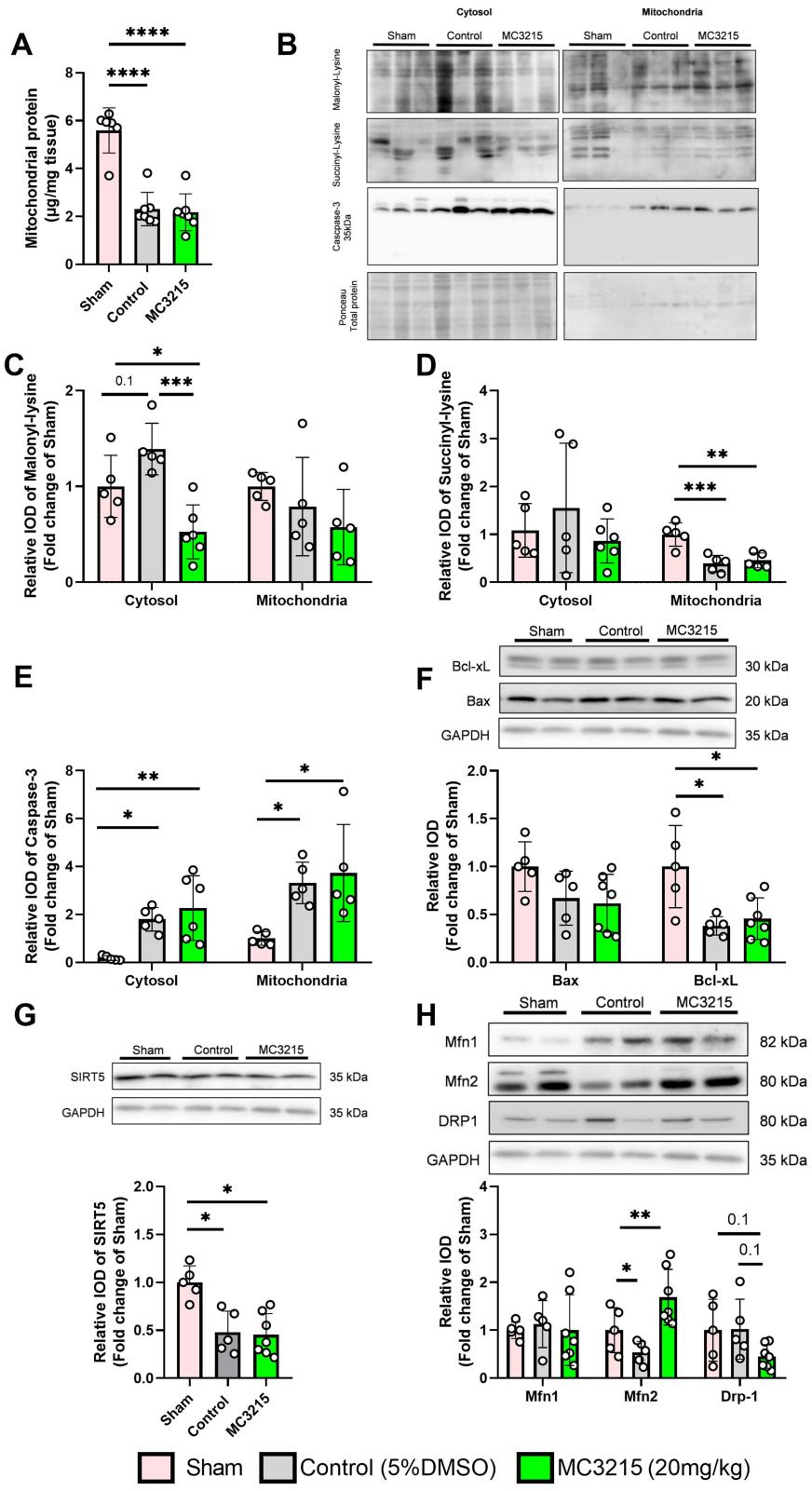

Figure 4 SIRT5 activation increases Mfn2, suggesting increased mitochondrial fusion. (**A**) Bar graph of total mitochondrial content per mg tissue. (**B**) Representative Western Blot images and relative densitometry graph of (**C**) malonyl-lysine, (**D**) succinyl-lysine, and (**E**) caspase-3 normalized to total protein after 3 h reperfusion in the cytosolic and mitochondria fractions. (**F**) Representative Western Blot images, and relative densitometry graph after 3h reperfusion of Bax and Bcl-xL, normalized to GAPDH. (**G**) RepresentativeWestern Blot image and relative densitometry graph of SIRT5 expression, normalized to GAPDH after 3 h reperfusion. (**H**) Representative Western Blot images and relative densitometry graphs after 3 h of reperfusion of the mitofusion-related proteins, namely Mfn1, Mfn2, and DRP1, normalized to GAPDH. Proteins run in the same SDS-PAGE gel, share the same GAPDH asa loading control. One-way ANOVA with Tukey's test was performed (*P< 0.05, **P< 0.01, ***P< 0.001, ****P < 0.0001).

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proteins, no significant differences were found among groups (Figure 4B and D). Succinylation in mitochondrial proteins was significantly reduced in the Control and MC3215 groups compared to Sham-operated animals, indicating that the SIRT5 agonist does not impact protein succinylation (Figure 4B and D).

We further investigated whether SIRT5 activation via MC3215 could regulate mitochondria-related apoptotic molecules in vivo. We focused on caspase-3 and cleaved caspase-3 levels at 3h of R: cleaved caspase-3 was not detected neither in the cytosolic nor in the mitochondrial fraction (Figure 4B). A statistically significant increase in full-length caspase-3 at 35 kDa was observed both in the Control and MC3215 groups compared to Sham-operated animals, equally in the cytosolic and mitochondrial fractions. Thus, it seems that short-term full-length caspase-3 regulation, at 3 h of reperfusion, does not contribute to MC3215-induced cardioprotection (Figure 4B and E). Moreover, in the mitochondrial fraction, we found no significant change in Bax expression, but a significant decrease in the expression of the anti-apoptotic factor Bcl-xL compared to Sham-operated mice, with no difference observed between the control and the MC3215-treated mice (Figure 4F), verifying that at this particular timepoint MC3215-induced cardioprotection seems to be independent of mitochondria-related apoptosis.

Since genetic silencing of SIRT5 has been previously shown to lead mitochondrial fragmentation and degradation,[26,27] we examined the expression of SIRT5 and whether MC3215 could regulate mitochondrial dynamics. Molecular analysis of the isolated mitochondria showed that SIRT5 expression is reduced in the control group compared to Sham, and MC3215 did not alter SIRT5 expression at 3 h of R (Figure 4G). Regarding mitochondrial dynamics, Mitofusin 1 (Mfn1) expression was not altered among groups, while Mitofusin 2 (Mfn2) was significantly increased by MC3215, which conversely tended to decrease the expression of Dynamin-related Protein 1 (Drp1) (Figure 4H). Collectively, our results demonstrate that the SIRT5 activation via MC3215 does not alter mitochondrial proteins related to apoptosis but may promote mitochondrial fusion through Mfn2.

SIRT5 Inhibition by TW-37 Abrogates the Cardioprotective Potential of SIRT5 Activation in vivo

To establish the role of pharmacological SIRT5 activation as a novel cardioprotective strategy, we employed the SIRT5 inhibitor TW-37[29] in the same in vivo model of 30 min I/2h R. Our results show that the co-administration of MC3215 and TW-37 abolished the cardioprotective effect of MC3215 (Figure 5A–C), confirming the causal role of SIRT5 pharmacological activation in MC3215-mediated cardioprotection.

Binding Prediction of MC3215 to SIRT5

To determine a potential binding geometry for the novel SIRT5 activator MC3215, molecular simulations were performed. Based on the hypothesis that an activator requires the presence of the substrate to exert its function, the SIRT5 structure (PDB ID: 8Z55) containing ADP and a succinylated substrate was used, providing a more complete representation of the active site.

For validation purposes, ADP was successfully redocked into its native position (Supplementary Figure 6A). To further refine the model, a ribose group from PDB structure 4F56 was incorporated into the 8Z55 structure. As an additional validation step, the well-known SIRT5 activator resveratrol was docked. After testing several parameter sets, top-ranked poses were identified in which resveratrol docked closely to the geometry observed in the crystallographic structure PDB ID: 4HDA (Supplementary Figure 6B), thereby validating the model.

MC3215 was then docked into the ADP-ribose–modified 8Z55 structure. The top-ranked result showed the thiophene group positioned near a ribose hydroxyl group, suggesting the formation of a potential chalcogen bond between the thiophene sulfur and the ribose oxygen (Figure 6A). To further investigate this interaction, quantum mechanical calculations were performed. A rigid scan of interaction energies across distances ranging from 3 to 10 Å revealed an energy difference of >2 kcal/mol, supporting the existence of a possible chalcogen bond (Figure 6B).

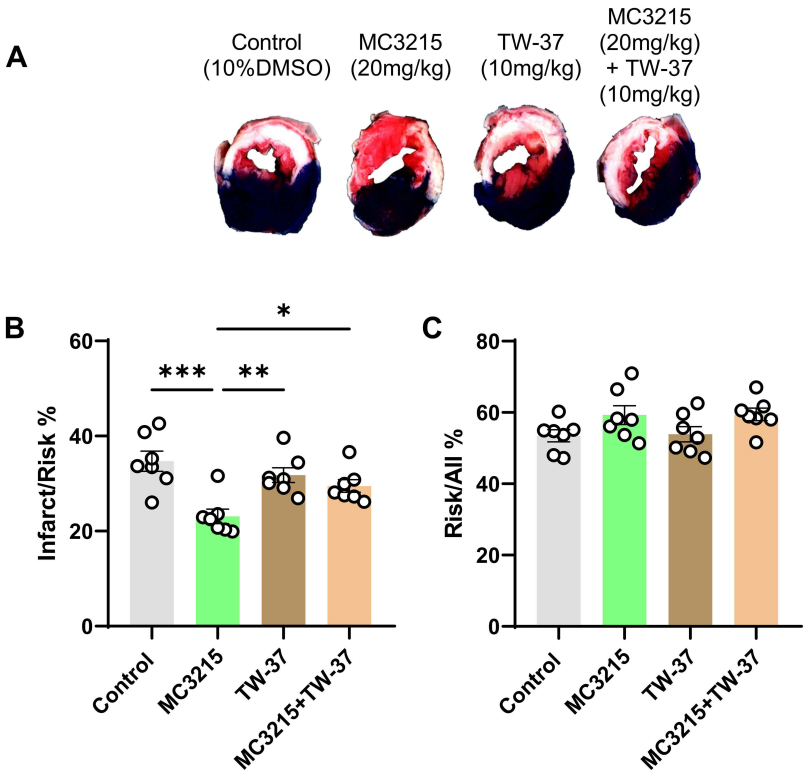

Figure 5 TW-37 abrogates the cardioprotective potential of the SIRT5 agonist MC3215 in an in vivo model of IRI. (**A**) Representative images of the myocardial infarcts are depicted. Heart slices after 30 min ischemia and 2 h reperfusion at 20 mg/Kg for MC3215 and 10 mg/Kg for TW-37. (**B**) The effects of MC3215 and TW-37 on IS are expressed as a percentage of the area at risk (% Infarct/Risk), and (**C**) the area at risk is expressed as a percentage of the total left ventricle area (% Risk/All) (n=7 per group, mean ± SD, One way ANOVA with Tukey's test was performed,*P< 0.05, **P< 0.01, ***P < 0.001 vs the Control group).

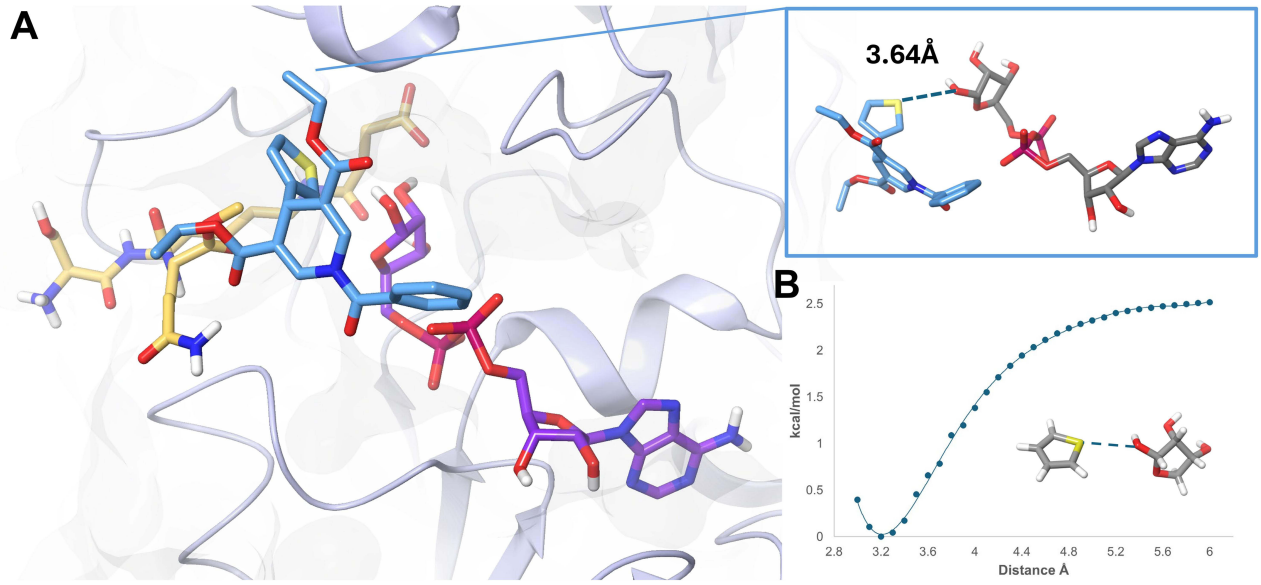

Figure 6 Docking of MC3215 and quantum mechanical analysis of potential chalcogen bonding. (**A**) Docked pose of MC3215 in the ADP-ribose–modified 8Z55 structure. As shown in the inlet, the thiophene group is positioned near the ribose hydroxyl group, suggesting a potential chalcogen bond between the sulfur atom of the thiophene and the oxygen atom of the ribose. (**B**) Quantum Mechanical rigid coordinate scan showing the interaction energy as a function of distance between the sulfur and oxygen atoms. The plot indicates an energetically favorable interaction at ~3.6 Å >2 kcal/mol, supporting the possibility of a chalcogen bond.

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Discussion

Herein, we demonstrate that the newly synthesized SIRT5-specific agonist, MC3215, protects H9c2 and EA.hy-296 endothelial cells against H/R injury and that in vivo administration of MC3215 at the 20th min of reperfusion reduces IS. Our results indicate, for the first time, that pharmacological activation of SIRT5 is a novel cardioprotective strategy and that AMPKα and RISK pathway activation are linked to the MC3215-cardioprotective effect.

SIRT1 MC2606 and SIRT5 MC3215 agonists exerted cytoprotective potential, observed at a concentration of 1 μM, highlighting the efficacy of these compounds at relatively low doses. SRT1720, the reference SIRT1 agonist, exhibited cytotoxicity at the same concentrations, although it was previously found to ameliorate IRI.[25] This is likely due to the higher doses tested, underscoring its narrow therapeutic range and highlighting the potentially safer profiles of MC2606 and MC3215. Our in vivo studies further demonstrated that the SIRT5-specific agonist MC3215 substantially reduced IS in anesthetized mice at a lower dose compared to the SIRT1 agonist and for 24 h of reperfusion. Our results are in agreement with a study demonstrating that IS was significantly increased in Sirt5$^{-/-}$ mice hearts relative to WT.[11] To our knowledge, no SIRT5 agonist has been tested either in vitro or in an in vivo model of IRI, marking our work as a significant and innovative contribution to the field.

The addiction of acyl groups to protein lysine residues (acylation) is a biologically significant post-translational modification.[43] Although many such acylations exist (eg, succinylation, glutarylation, and malonylation), in our research approach, we focused on malonylation, since SIRT5 is the only demalonylating sirtuin, and we also provide some insights on lysine desuccinylation upon MC3215 administration. The results of the present study collectively suggest that malonylation is not significantly affected by IRI up to 3 hours of reperfusion, hence SIRT5 agonist MC3215 decreases malonylation, exerting cardioprotective potential. This effect can either suggest that demalonylation is not essential for cardioprotection or that the metabolic swifts afforded by MC3215 are not dependent on IRI-specific mechanisms. In patients with ST-segment elevation myocardial infarction (STEMI), the levels of succinylation and glutarylation, and not malonylation, are significantly reduced in the peripheral serum compared to healthy controls, but information regarding myocardial malonylation in myocardial IRI is scarce.[44] Conversely, malonyl-CoA decarboxylase inhibition, which suppresses fatty acid oxidation, improves heart failure in a rat model of LAD permanent ligation.[45]

MC3215 did not affect succinylation, while at 3 h of reperfusion, we found that mitochondrial succinylation was reduced by IRI at 3 h of reperfusion. Kathleen A. Hershberger et al, 2018,[46] reported a tamoxifen-induced ablation of SIRT5 in a mouse model in which SIRT5 was depleted within 3 weeks upon tamoxifen treatment, while protein succinylation in the heart lysates continued to increase for at least 30 weeks, suggesting that succinylation modifications require time to accumulate.

Recently, the SIRT5 isoform has gained increasing attention for its multiple roles in metabolism, affecting glycolysis and FAO.[27] AMPK and SIRTs are partner proteins coordinating numerous intracellular processes, including metabolism. The effect of SIRT5 on AMPK activation is controversial. In support of our findings, SIRT5 overexpression increased AMPK phosphorylation during the differentiation of preadipocytes.[47] On the other hand, SIRT5 deficiency promoted AMPK activation in mice with transverse aortic constriction-induced cardiac hypertrophy,[48] while in arterial tissue, Sirt5$^{-/-}$ mice displayed increased phosphorylation of AMPK, leading to blunted fibrinolysis.[49] In our setting of IRI, SIRT5 pharmacological agonism via MC3215 led to AMPKα activation via phosphorylation, and our metabolomic analysis pointed out metabolic mediators related to FAO. AMPK activation in early reperfusion is associated with a reduction in IS, and its activation is beneficial for the ischemic heart.[50] Our findings are consistent with other studies highlighting the cardioprotective effects of the molecules inducing AMPK-FAO. For instance, the AMPK activator AICAR (5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside) was found to improve FAO in cardiac tissues in models of cardiotoxicity, such as those induced by doxorubicin, therefore improving cardiac function.[51] Taken together with the metabolomics analysis and AMPK activation, our data support that SIRT5 activation may promote metabolic flexibility towards FAO.

The RISK pathway is crucial in protecting the heart from IRI, primarily by activating pro-survival kinases that reduce apoptosis and necrosis in cardiomyocytes.[36] We have demonstrated that MC3215 activates this crucial cardioprotective pathway, particularly enhancing the expression of Akt, eNOS, and GSK3β, suggesting that it activates protective

signaling cascades. The crosstalk between SIRT5, Akt, GSK3β, and AMPK signaling has not been elucidated. However, in cardiac myocytes and in vascular endothelial cells, Akt activation appears to be regulated by AMPK activation.[52]

MC3215 increased the expression of Mfn2 and tended to decrease Drp1 expression, suggesting a shift towards enhanced mitochondrial fusion, which is associated with improved mitochondrial function and cellular resilience.[26] This is particularly relevant in the context of cardiac IRI, where maintaining mitochondrial integrity is crucial for cell survival and function.[10,53] Mdivi-1, a Drp1 inhibitor, has been shown to improve mitochondrial function and reduce IS in models of cardiac IRI, leading to preserved mitochondrial dynamics and enhanced cardiomyocyte survival.[42] Moreover, SIRT5-mediated inhibition of Drp1 hampers its translocation to the mitochondrial membrane, consequently preventing mitochondrial fragmentation.[10,26] Mitochondrial fusion has also been observed upon activation of AMPK. Metformin, an AMPK activator, ameliorated IR-induced retinal damage by up-regulating the expression of Mfn2 and OPA1 in dependence on the AMPK pathway, which preserved mitochondrial fusion, and alleviated IRI.[54] Activation of AMPK prevents and reverses mitochondrial hepatocellular injury via restoration of fusion and enhanced autophagy-mediated removal of damaged mitochondria.[55] Yet, we did not analyze mitochondrial membrane potential upon treatment with MC3215, and this mechanism needs further investigation. However, MC3215 did not influence the levels of apoptotic molecules, including full-length and cleaved caspase-3 and the anti-apoptotic factor Bcl-xL, contrasting with previous studies that showed SIRT5 enhances Bcl-xL expression in cardiomyocytes.[9] Those studies involved overexpressing SIRT5 or using SIRT5 knockdown models without directly testing SIRT5 agonists, which is more pharmacologically relevant. However, we must note that at the selected timepoint of 3 h of reperfusion, at which changes in mitochondrial fission have crescented,[56] caspase 3 cleavage and induction of apoptosis are minimal.[57,58] Conclusively, our results indicate that during early reperfusion up to 3 h, mitochondrial-related apoptosis does not seem to be the primary mechanism of MC3215 cardioprotection, whilst alternative mechanisms of cardioprotection including metabolic- and mitochondrial dynamics-related pathways primarily contribute to MC3215 cardioprotection. The contribution of apoptosis and the impact of MC3215 on long-term inhibition of cellular death cannot be ruled out and will be subsequently investigated in in vivo models of heart failure with reduced ejection fraction (HFrEF).

Several studies highlighted the crucial role of SIRT5 in maintaining redox homeostasis by modulating the activity of antioxidant enzymes, thereby reducing oxidative stress and protecting cells from ROS-induced damage.[8,27] MC3215 did not influence ROS production or lipid peroxidation. A limitation of our study is that we examined ROS in the cardiac tissue and did not measure ROS production on isolated mitochondria. Therefore, future studies could further elucidate the effect of SIRT5 pharmacological activation on oxidative stress regulation. Although we aimed to provide high translational value in our in vivo protocols, certain limitations should be acknowledged. The investigation of the cardioprotective effect of MC3215 is limited by the fact that dose escalation studies were not performed and causal relationships among the involved pathways were not explicitly deciphered.

Conclusively, to determine the causal role of SIRT5 activation in cardioprotection, we co-administered MC3215 with the SIRT5 inhibitor TW-37.[29] The cardioprotective effect of MC3215 was abolished, indicating that the infarct-sparing potential is SIRT5-dependent. The administration per se of TW-37 did not lead to increased myocardial IS, suggesting that SIRT5 antagonism can be compensated by the reperfused myocardium. Hence, the SIRT5 inhibitor, TW-37, has also been described to inhibit Bcl-2, a protein regulating apoptosis,[34] potentially complicating the interpretation of the outcomes. Regarding the target engagement of MC3215 to SIRT5 via docking calculations, our analysis was based on the only available crystallographic structure of an activator bound to SIRT5 is 4HDA,[59] which features resveratrol in the binding site. Although the electron density for resveratrol in this structure is relatively low, it served as the only available reference and was therefore used to guide the development of a docking model suitable for evaluating potential binding modes of MC3215. Resveratrol was co-crystallized alone with the protein, but we hypothesized that it should bind together with the substrate in order to exert its activating effect, and therefore we have built our theoretical model also by co-positioning ADP-ribose. Building on our refined model, the docking of MC3215 revealed a compelling interaction between its thiophene moiety and the ribose hydroxyl group, with geometric features consistent with a potential chalcogen bond. Such non-covalent interaction, although relatively weak, can significantly contribute to binding affinity and specificity. The predicted chalcogen bond offers a mechanistic rationale for MC3215's binding orientation, but future

experimental validation, such as site-directed mutagenesis or NMR spectroscopy, could further confirm the presence and relevance of this interaction in solution.

This study has several limitations. Only male mice were used, and therefore, the generalizability of findings in both sexes may be limited. Moreover, we did not use inhibitors for each target in the described pathways to determine causal relationships besides SIRT5 activation. The results of the present study should be verified in a more relevant-to-humans large animal model prior to translation.

Conclusions

Collectively, these findings highlight SIRT5 as a promising therapeutic target for cardioprotection against IRI. SIRT5 activation via MC3215 enhanced cell viability and reduced IS, rendering MC3215 a potent pharmacological tool/intervention. MC3215 given at a translational timepoint (exactly prior to reperfusion) serves as a per-condition mimicking strategy, activates endogenous cardioprotective pathways, and preserves mitochondrial dynamics, encouraging future research on SIRT5 activation in the context of ischemic heart disease.

Abbreviations

Akt, Protein Kinase B; AMPK, AMP-activated Protein Kinase; ATP, Adenosine Triphosphate; Bax, BCL2 associated X protein; Bcl-xL, B-cell lymphoma-extra-large; DHE, Dihydroethidium; DMSO, Dimethyl Sulfoxide; Drp1, dynamine-related protein 1; eNOS, Endothelial Nitric Oxide Synthase; FAO, Fatty Acid Oxidation; GSK-3β, glycogen synthase kinase-3 beta; H/R, hypoxia/reoxygenation; I, ischemia; IRI, ischemia/reperfusion injury; IS, infarct size; LAD, descending coronary artery; MDA, malondialdehyde; Mfn1, mitofusin 1; Mfn2, mitofusin 2; MI, Myocardial Infarction; MnSOD, superoxide dismutase 2; MS, Mass Spectrometry; NAD, nicotinamide adenine dinucleotide; PCA, principal component analysis; PCI, percutaneous coronary intervention; R, reperfusion; RISK, Reperfusion Injury Salvage Kinase; ROS, reactive oxygen species; SD, standard deviation; SIRT, sirtuin; STAT3, signal transducer and activator of transcription 3; TTC, Triphenyl-Tetrazolium Chloride.

Data Sharing Statement

All data are available upon request to the corresponding authors.

Acknowledgments

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This article is dedicated to the memory of Professor Ioanna Andreadou, whose untimely passing during its preparation was a profound loss to the scientific community. This work shall commemorate her scientific oeuvre and her substantial impact in the field of cardioprotection. Above all, the authors wish to honour her for her kindness, generosity, and unwavering passion for science and beyond.[61]

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors report that there are no conflicts of interest in this work.

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