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Inhibition of the proteasome preserves Mitofusin-2 and mitochondrial integrity, protecting cardiomyocytes during ischemia-reperfusion injury

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ABSTRACT

Cardiomyocyte loss is the main cause of myocardial dysfunction following an ischemia-reperfusion (IR) injury. Mitochondrial dysfunction and altered mitochondrial network dynamics play central roles in cardiomyocyte death. Proteasome inhibition is cardioprotective in the setting of IR; however, the mechanisms underlying this protection are not well-understood. Several proteins that regulate mitochondrial dynamics and energy metabolism, including Mitofusin-2 (Mfn2), are degraded by the proteasome. The aim of this study was to evaluate whether proteasome inhibition can protect cardiomyocytes from IR damage by maintaining Mfn2 levels and preserving mitochondrial network integrity. Using *ex vivo* Langendorff-perfused rat hearts and *in vitro* neonatal rat ventricular myocytes, we showed that the proteasome inhibitor MG132 reduced IR-induced cardiomyocyte death. Moreover, MG132 preserved mitochondrial mass, prevented mitochondrial network fragmentation, and abolished IR-induced reductions in Mfn2 levels in heart tissue and cultured cardiomyocytes. Interestingly, Mfn2 overexpression also prevented cardiomyocyte death. This effect was apparently specific to Mfn2, as overexpression of Miro1, another protein implicated in mitochondrial dynamics, did not confer the same protection. Our results suggest that proteasome inhibition protects cardiomyocytes from IR damage. This effect could be partly mediated by preservation of Mfn2 and therefore mitochondrial integrity.

1. Introduction

Acute myocardial infarction is a leading cause of death around the world. During ischemia, a dwindling supply of oxygen and nutrients provokes events that may ultimately lead to cell death. Rapid reperfusion is essential for salvaging cardiac tissue; however, this process itself may promote further damage. These events are generally referred to as myocardial ischemia-reperfusion (IR) injury [1–3].

Mitochondrial dysfunction plays a key role in IR-induced cardiomyocyte death and has therefore become a primary cellular target for minimizing IR injury [4]. Mitochondria are responsible for approximately 90% of ATP production in cardiomyocytes, and their function is tightly modulated by fusion and fission processes [5,6]. Drp1 and Fis1 proteins are involved in mitochondrial fission, which is associated with a fragmented mitochondrial phenotype. The fusion process, on the other hand, is characterized by long, interconnected mitochondrial networks and is regulated by Mitofusin-1 and -2 (Mfn1 and Mfn2) and Opa1 [7]. Other mitochondrial proteins are involved in mitochondrial transport and dynamics. One such protein, Miro1, is an atypical Rho GTPase located in the outer mitochondrial membrane. Miro1 plays a pivotal role in calcium-induced regulation of mitochondrial motility in neurons. Miro1 may also interact with Mfn2 in neurons

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[8,9]. During IR injury, cardiomyocytes eliminate dysfunctional or aged mitochondria through autophagy (mitophagy) after the fission process is complete [10,11]. However, uncontrolled mitochondrial fission can exacerbate IR injury [12]. Drp1 inhibition has been shown to reduce cardiomyocyte death after simulated IR (sIR) injury and attenuates myocardial infarct size in adult mouse and rat hearts [13–15]. The role of mitofusins is less well-understood. *In vitro* studies have shown that Mfn2 overexpression protects against sIR-induced HL-1 cell death, suggesting a cardioprotective role for this protein in cellular models of acute IR injury [13]. However, other studies have reported that cardiac-specific ablation of Mfn1 and Mfn2 [16] or Mfn2 alone [17] has a protective effect against myocardial infarction in the adult heart.

Proteasomes are key enzymes in the ubiquitin-proteasome system (UPS). The UPS is the main protein degradation pathway in the heart and is essential for cellular protein homeostasis [18]. The UPS eliminates misfolded or damaged proteins, which are selectively poly-ubiquitinated, recognized, and then degraded by the proteasome [19]. Moreover, inhibition of the proteasome exerts a cardioprotective effect in various models. Proteasome inhibition protects neonatal cardiomyocytes from anoxia/reperfusion-induced necrotic death by preventing neutrophil extracellular trap formation [20]. In adult hearts, the proteasomal inhibitor MG132 prevents degradation of connexin-43 or caveolin-3 during heart failure [21] or IR [22] in rat hearts, respectively. Likewise, in a hypertrophic cardiomyopathy model involving mice with a myosin-binding protein C mutation, cardiac function increased after partial inhibition of the chymotrypsin-like activity of the proteasome [23]. The mechanisms by which proteasome inhibition induces cardioprotection remain to be fully elucidated, but it appears that the protection conferred may depend on the concentration of the inhibitor [24].

Interestingly, it has been shown that both at baseline and during mitophagy, Mfn1 and Mfn2 are degraded by the proteasome, inducing fission and mitochondrial degradation [25–27]. Therefore, based on our previous studies and the literature, the aim of this work was to evaluate whether proteasome inhibition protects mitochondrial integrity after IR by preserving Mfn2 protein content in cultured cardiomyocytes. Our results show that proteasome inhibition protects against IR injury in the heart at least in part by maintaining Mfn2 protein levels, suggesting a crucial role for Mfn2 in preserving cardiomyocyte viability during IR.

2. Materials and methods

2.1. Animals and primary cultures of neonatal rat ventricular myocytes

All animal experiments were approved by the Animal Care and Use Committee of the University of Chile and conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication, 8th Edition, 2011).

Adult male (220–240 g) and neonatal Sprague-Dawley (1–3 days old) rats were used for IR experiments. Briefly, neonatal rat ventricular myocytes (NRVMs) were obtained from neonatal rats euthanized by decapitation. The hearts were quickly harvested, and ventricles were digested using pancreatin (1 μg/mL, Sigma-Aldrich Corp, St Louis, MO, USA). Cells were pre-plated to discard non-myocyte cells. The myocyte-enriched fraction was plated at 1.0×10^6 cells/mm^2 on gelatin-precoated 35-mm dishes (Falcon, BD Biosciences, Oxford, UK) and maintained in Dulbecco's modified Eagle medium (HyClone, Logan, UT, USA) and M199 medium (HyClone, DMEM:M199; ratio 4:1), with 10% (*w/v*) fetal bovine serum (FBS, HyClone), 5% (w/v) equine serum (HyClone), 100 mM 5-bromo-2′-deoxyuridine (Sigma-Aldrich), and penicillin/streptomycin (100 U/mL/100 μg/mL, HyClone) for 24 h at 37 °C in an incubator containing 95% air and 5% CO_2 until use in experiments. NRVMs were at least 95% pure as evaluated by immunofluorescence using an anti-β-myosin heavy chain antibody (Vector Laboratories, Burlingame, CA, USA) [28].

2.2. Ex vivo ischemia/reperfusion

Adult male rats were anesthetized with pentobarbital (80 mg/kg, intraperitoneal), and heparin (100 U/kg) was injected into the right atrium. As described previously [24], hearts were rapidly excised and perfused at 37 °C *via* the ascending aorta, with a Krebs-Henseleit (KH) solution containing (in mM): 128.3 NaCl, 4.7 KCl, 1.35 $CaCl_2$, 1.1 $MgSO_4$, 20.2 $NaHCO_3$, 0.4 NaH_2PO_4, and 11.1 glucose; pH 7.4. KH buffer was equilibrated with a gas mixture of air (95%) and CO_2 (5%) and perfused at a constant flow of 10–14 mL/min. After 20 min of stabilization, hearts were subjected to 30 min of global ischemia at 37 °C and 60 min of reperfusion with KH oxygenated solution. The proteasome inhibitor MG132 (Merck Millipore, Billerica, MA, USA) was perfused at 0.5 or 6 μM only before global ischemia. Control hearts not subjected to ischemia were perfused with or without MG132 for 110 min. Left ventricular hemodynamic parameters were measured with a ventricular latex balloon connected to a pressure transducer. At the end of the experiments, hearts were snap-frozen in liquid N_2 or perfused with triphenyl tetrazolium 1% (TTC, 1%) to determine infarct size. Briefly, hearts were perfused with TTC in phosphate buffer (pH 7.4) for 10 min and immersed in this solution for another 10 min, frozen at −20 °C for 1 h, and then cut into six transverse slices using a chopper. After 24 h of fixation in 10% formaldehyde solution, the slices were digitally photographed. The infarct area was calculated by planimetry and expressed as a percentage of the total area of the heart.

2.3. In vitro simulated ischemia-reperfusion

As described previously [29], ischemia was performed in NRVMs with an ischemic medium containing (in mM): HEPES (5), 2-deoxy-D-glucose (10), NaCl (139), KCl (12), $MgCl_2$ (0.5), $CaCl_2$ (1.3), and lactic acid (20); pH 6.2. NRVMs were then placed within a humidified gas chamber equilibrated with O_2 (<1%), CO_2 (5%), and N_2 (94–95%) and kept at 37 °C for 6 h. Reperfusion was initiated by changing the ischemia-mimicking solution to DMEM:M199 (4:1) supplemented with 10% (*w/v*) FBS in normoxia (95% air and 5% CO_2) for 16 h at 37 °C. Parallel controls were similarly incubated in normoxic conditions with control buffer containing (in mM): HEPES (5), D-glucose (23), NaCl (139), KCl (4.7), $MgCl_2$ (0.5), $CaCl_2$ (1.3), pH 7.4. NRVMs were "reperfused" by replacing the buffer with the same media as cells subjected to simulated ischemia-reperfusion (sIR).

2.4. NRVM transduction and inhibitors

To overexpress Mitofusin-2 (Mfn2) or Miro1 or to knockdown Mfn2 expression in NRVMs, cells were transduced with the corresponding adenovirus 36 h after plating at a multiplicity of infection (MOI) of 1 and 50 (Mfn2); 10, 20 and 50 (Miro1) or 300 (Mfn2), respectively. Empty vector (LacZ to Mfn2 or CMV to Miro1) was used as a control for transduction. Experiments were performed 12 h after adenoviral transduction. MG132 (2, 4, and 8 μM) and bafilomycin A1 (BA, 50 nM) were obtained from Calbiochem (La Jolla, CA, USA) and Sigma-Aldrich, respectively, and used 30 min before and during the ischemic stimuli.

2.5. Proteasome activity

Proteasome activity was determined as described previously [24]. Briefly, homogenates from rat hearts or NRVMs were incubated in the presence of fluorogenic proteasome substrates: Suc-LLVY-amc (21 μM) for chymotrypsin-like activity, *Z*-LLE-amc (105 μM) for caspase-like activity, and Boc-LSTR-amc (34 μM) for trypsin-like activity (Sigma-Aldrich). Fluorescence was measured at 30 °C in a plate reader at 380-nm excitation/440-nm emission wavelengths. Non-specific proteolysis was determined in the presence of MG132 (30 μM).

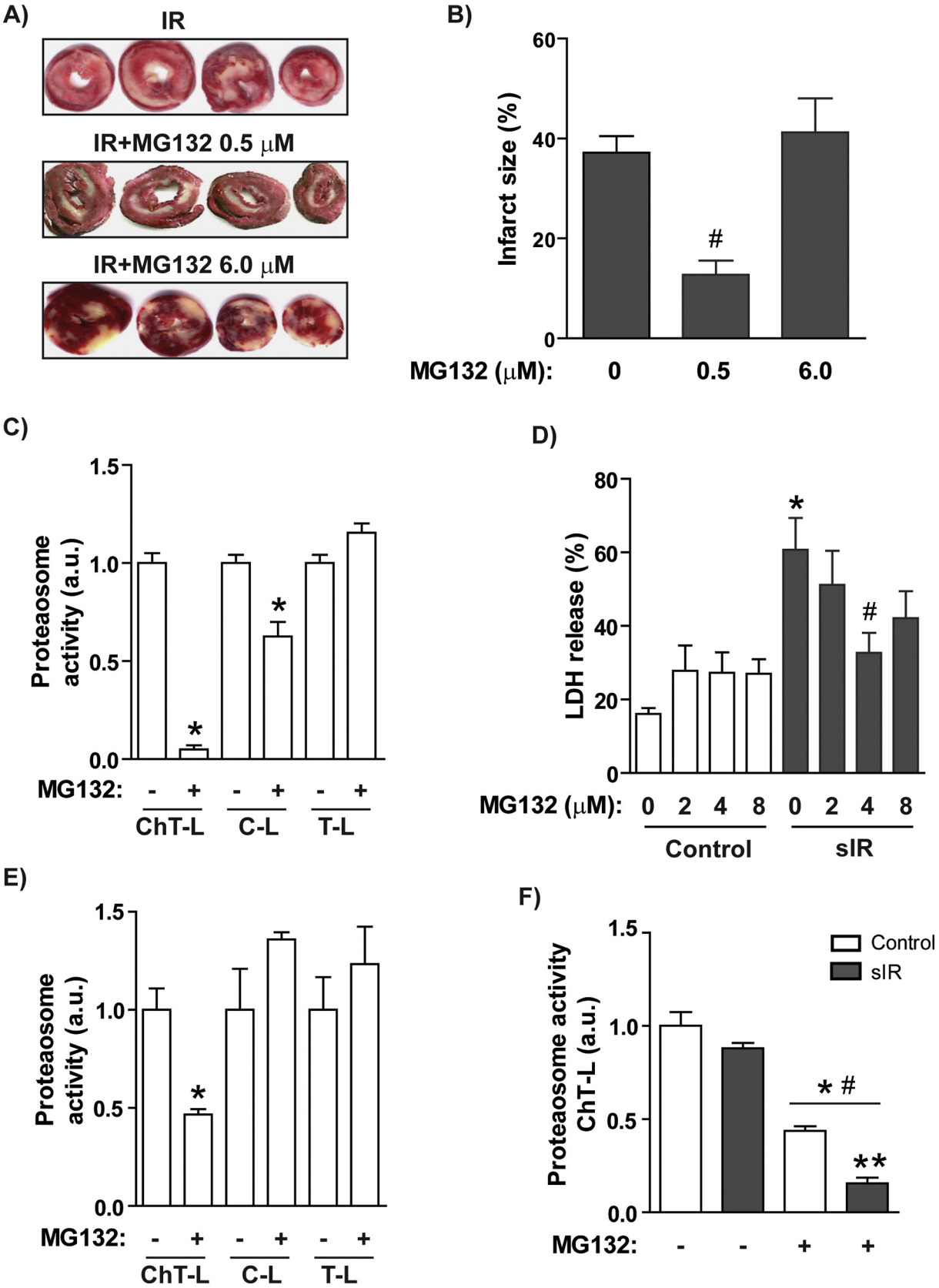

Fig. 1. Proteasome inhibition in cardiomyocytes prevents IR-induced injury. A) Representative heart slices stained with TTC after IR and in the presence of MG132 at 0.5 and 6 μM. B) Infarct size as a % of total cardiac area ($n = 6$). C) Proteasome activity in control tissue samples (ChT-L: chymotrypsin-like; C-L: caspase-like and T-L: trypsin-like), ($n = 4$). D) Percentage of lactate dehydrogenase (LDH) released into the culture medium (n = 4–7). E) Proteasome activity in control NRVMs in the presence of 4 μM of MG132 ($n = 3$). F) Chymotrypsin-like activity in control NRVMs and after sIR in the presence of MG132 (4 μM, n = 3). Values are shown as mean ± SEM. *$p < .05$ *vs.* control, #vs. IR and **vs. C + MG132.

2.6. Western blot analysis

Proteins were obtained from rat hearts and NRVMs using a cold T-PER buffer (Thermo Scientific, Rockford, IL, USA) in the presence of a protease and phosphatase inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Proteins were separated by SDS-PAGE (8 or 15% gels), transferred to PVDF (Millipore Corp) membranes, and immunoblotted. The primary antibodies used were: anti-Mfn2 and anti-Mfn1 (Abcam, Cambridge, MA, USA), anti-LC3B (Cell Signaling, Danvers, MA, USA), anti-Miro1 (Abcam), and anti-GAPDH (Sigma-Aldrich). Antigen-antibody reactions were detected by EZ-SL (Biological Industries, USA), and blots were quantified using Image Lab software. Results were normalized to GADPH.

2.7. Mitochondrial DNA extraction and qRT-PCR

To extract DNA, NRVM was homogenized with DNAzol® reactive (Invitrogen, Carlsbad, CA, USA), and the samples were centrifuged at 10,000 xg for 10 min at 4 °C. Total DNA was precipitated from the supernatant with ethanol and quantified. Mitochondrial DNA (mtDNA) was quantified by detecting D-loop with PCR primers, as previously described [30]: F: 5'GGT TCT TAC TTC AGG GCC ATC A-3′ and R: 5′-GAT TAG ACC CGT TAC CAT CGA GAT-3′. β-actin, used as a housekeeping gene, was detected with specific primers: F: 5′-GGG ATG TTT GCT CCA ACC AA-3′ and R: 5'-GCG CTT TTG ACT CAA GGA TTT AA-3′. The ΔCt method was used to calculate relative transcript abundance.

2.8. Mitochondrial dynamics analysis

After a shorter period of ischemia (2 h) and reperfusion (1 h), NRVMs were incubated on coverslips for 30 min with MitoTracker Green FM (400 nM, Thermo Scientific, Rockford, IL, USA) and maintained in Krebs solution. Confocal image stacks (fifteen 45-nm stacks per cell) were obtained with a Zeiss LSM-5, Pascal 5 Axiovert 200 microscope, using LSM 5 3.2 image capture and analysis software as previously described [31]. Images were deconvolved with Image J (NIH), and z-stacks of threshold images were volume-reconstituted using the VolumeJ plug-in. The ImageJ-3D Object counter plug-in was used to quantify the number and volume of individual mitochondria. Mitochondrial fission was quantified as the mean decrease in mitochondrial volume in the presence of an increased number of mitochondria. Each experiment was performed at least 4 times, and 4–8 cardiomyocytes per condition were quantified.

2.9. Other procedures

Lactate dehydrogenase (LDH) activity was measured using the CytoTox 96®, Non-Radioactive Cytotoxicity Assay kit (Promega Corporation WI, USA), according to the manufacturer protocol. Proteins were determined using Quick Start™ Bradford Dye reagent (Bio-Rad), following the manufacturer's instructions. Reactive oxygen species (ROS) production was measured using dihydrorhodamine 123 (DHR123, Sigma-Aldrich). Briefly, after 2 h of simulated ischemia and 1 h of reperfusion, NRVMs were treated with DHR123 (100 μM) for 30 min (during reperfusion) at 37 °C and 5% CO_2. NRVMs were detached using trypsin (Thermo Scientific, Rockford, IL, USA) and fluorescence was determined by flow cytometry using a FACScan system (Becton Dickinson).

2.10. Statistical analysis

Data are shown as the mean ± SEM of the indicated number (n) of independent experiments. For *ex vivo* protocols, each heart is an independent experiment, while for *in vivo* protocols, each culture is an independent experiment. Replicate experiments are from the same cardiomyocyte culture. Data were analyzed with GraphPad Prism 7.0 software, using Student's unpaired *t*-test or ANOVA followed by Tukey's test. Differences were considered significant at $p < .05$.

3. Results

3.1. Inhibition of the proteasome protects the heart from IR injury

To study the cardioprotective role of proteasome inhibition, MG132 (0.5 or 6 μM) was administered to isolated adult rat hearts subjected to 30 min of global ischemia, followed by 60 min of reperfusion. We observed that 0.5 μM of MG132 decreased the percentage of infarcted cardiac tissue as compared to untreated hearts, but 6 μM of the inhibitor did not prevent cardiac injury (Fig. 1A, B). To evaluate whether the proteasome remains inhibited throughout the whole experiment, we measured proteasome activity after reperfusion in control samples. Our results show that the chymotrypsin-like (ChT-L) and caspase-like (C-L) activities of the proteasome remained inhibited in the presence of 0.5 μM of MG132 at the end of the experiments (Fig. 1C), but trypsin-like activity levels (T-L) remained unaltered.

To investigate whether inhibition of the proteasome exerts a direct protective effect on cardiomyocytes, we treated NRVMs with 2, 4 or 8 μM of MG132 for 6 h of simulated ischemia followed by 16 h of reperfusion. Cell death was assessed by measuring lactate dehydrogenase (LDH) release into the culture medium. Our data show that sIR induced necrosis in NRVMs (Fig. 1D). Moreover, 4 μM of MG132 reduced cell death as compared to the untreated condition, whereas 2 and 8 μM had no effect on sIR-induced cardiomyocyte death (Fig. 1D). Also, our results suggest that all three MG132 concentrations tested decreased the chymotrypsin-like (ChT-L) activity of the proteasome in a dose-dependent manner after reperfusion in control NRVMs, but no changes in caspase-like (C-L) were observed. Tripsin-like (T-L) activity was inhibited only at 8 μM as compared with control condition. Fig. 1E shows the results for 4 μM of MG132 and Supplementary Fig. 1A shows the proteasomal activities with 2 to 8 μM of MG132. During sIR, ChT-L activity in the NRVMs decreased to levels below those of the controls in the presence of the inhibitor (Fig. 1F). Taken together, our results show that proteasome inhibition protects cardiomyocytes from IR injury. For the remaining experiments, we used 0.5 μM or 4 μM of MG132 for *ex vivo* and *in vitro* experiments, respectively.

3.2. Inhibition of the proteasome prevents Mitofusin-2 degradation

Next, we sought to study the role of the mitochondrial protein Mfn2 in the protective effect of proteasome inhibition, as this protein is known to be degraded by the proteasome and has a controversial role in cardioprotection under IR conditions. As shown in Fig. 1, proteasome inhibition protects against IR injury *ex vivo* and *in vitro*. We hypothesized that this protection could be due to preservation of Mfn2 levels and mitochondrial connectivity. We first determined Mfn2 protein levels after IR injury using Western blot. Mfn2 protein content was decreased after *ex vivo* IR (Fig. 2A) and *in vitro* sIR (Fig. 2B). These effects were prevented, at least in part, by inhibition of the proteasome with MG132 during *ex vivo* IR (Fig. 2A). Furthermore, the effects were completely prevented during *in vitro* sIR by 4 μM (Fig. 2B and Supplementary Fig. 1B), but not 2 or 8 μM, of MG132 (Supplementary Fig. 1B). Since Mitofusin-1 (Mfn1) is another mitochondrial fusion protein similar to Mfn2 [7], we measured Mfn1 protein levels during IR in the presence of MG132. Our results showed that Mfn1 protein levels did not change during *ex vivo* IR or *in vitro* sIR under baseline conditions or even in the presence of the proteasome inhibitor (Supplementary Fig. 1C–E). These results suggest that proteasome inhibition prevents Mfn2 degradation during IR in cardiomyocytes, without apparent changes in Mfn1 protein levels.

3.3. Autophagy is not involved in Mfn2 degradation during sIR

The proteasome and autophagy systems engage in communication at multiple points to regulate protein degradation and organelle homeostasis [32]; therefore, proteasome inhibition may regulate activation of autophagy, thereby inducing Mfn2 and mitochondrial degradation. Since MG132 did not recover Mfn2 protein content to normoxic levels during sIR, our results suggest that another mechanism could be involved in Mfn2 degradation in cardiomyocytes. To explore this possibility, we evaluated the role of autophagy. We first studied the potential link between proteasome degradation and autophagy by subjecting NRVMs to sIR and measuring LC3II protein levels. We observed an increase in LC3II content in response to sIR (Supplementary Fig. 2A). Furthermore, administration of MG132 did not alter this effect (Supplementary Fig. 2A). To confirm this finding, we measured autophagic flux in cardiomyocytes with the autophagy inhibitor bafilomycin A (BA, 50 nM) and/or MG132 and then measured LC3II content. Our data confirmed an increase in autophagy in the presence of BA, with or without MG132, as evidenced by increased LC3II levels (Supplementary Fig. 2B). These data suggest that autophagy is elevated during sIR and that autophagy levels are unchanged in the presence of MG132.

We also explored whether autophagy is involved in Mfn2 degradation during sIR. As shown previously, MG132 prevented Mfn2 degradation during sIR; however, BA completely inhibited the effects of MG132 on Mfn2 levels (Fig. 2C). Moreover, BA also prevented the increase in Mfn2 content in response to MG132 treatment under baseline conditions (data not shown), suggesting that BA either inhibited the effect of MG132 or increased proteasome activity. To test this question, we measured proteasome activity after co-incubating MG132 and BA. Our results show that MG132 inhibits the proteasome even in the presence of BA (Fig. 2D). Moreover, while MG132 alone inhibited only ChT-L activity, in the presence of BA, MG132 also inhibited proteasomal C-L and T-L activities (Fig. 2D).

3.4. Proteasome inhibition protects mitochondria from sIR

To assess whether inhibition of the proteasome preserves the mitochondria after IR, we measured mitochondrial DNA (mtDNA) after sIR in NRVMs using quantitative PCR. Our results show that sIR decreases mtDNA content, but MG132 is capable of completing abolishing this effect (Fig. 3A). Additionally, to study changes in mitochondrial

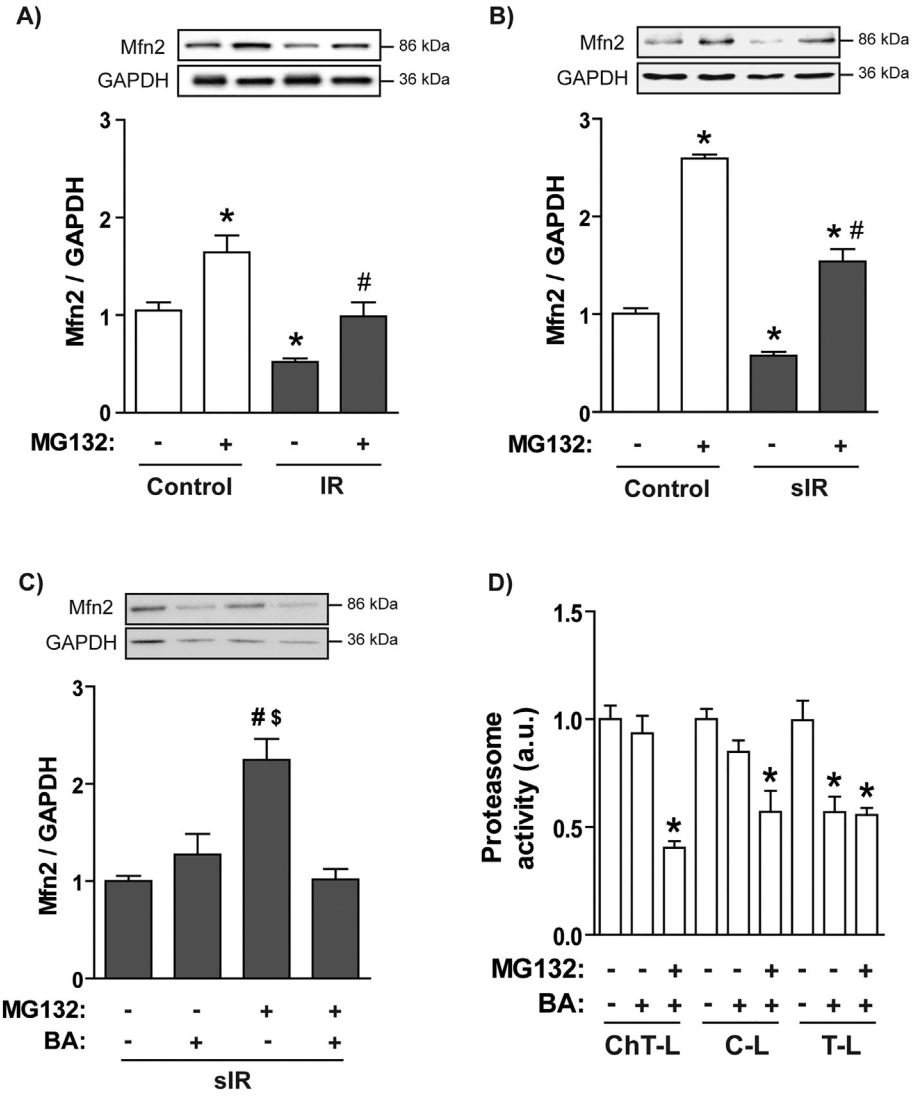

Fig. 2. Mitofusin-2 is degraded *via* the proteasome during IR, independent of the autophagy pathway. Representative Western blots of Mfn2 and GAPDH (loading control) in: A) Heart homogenates (0.5 μM, $n = 8–9$), B) NRVMs with or without MG132 (4 μM, $n = 4–5$), and C) NRVMs with or without MG132 plus bafilomycin A (BA) after sIR (n = 6–8). Graphs show the ratio of Mfn2/GAPDH. D) Proteasome activity in control NRVMs with BA and BA plus MG132 (ChT-L: chymotrypsin-like; C-L: caspase-like and T-L: trypsin-like) (n = 3). Values are shown as mean ± SEM. *$p < .05$ *vs.* control, #*vs.* IR or sIR and $*vs.* sIR + MG132 + BA.

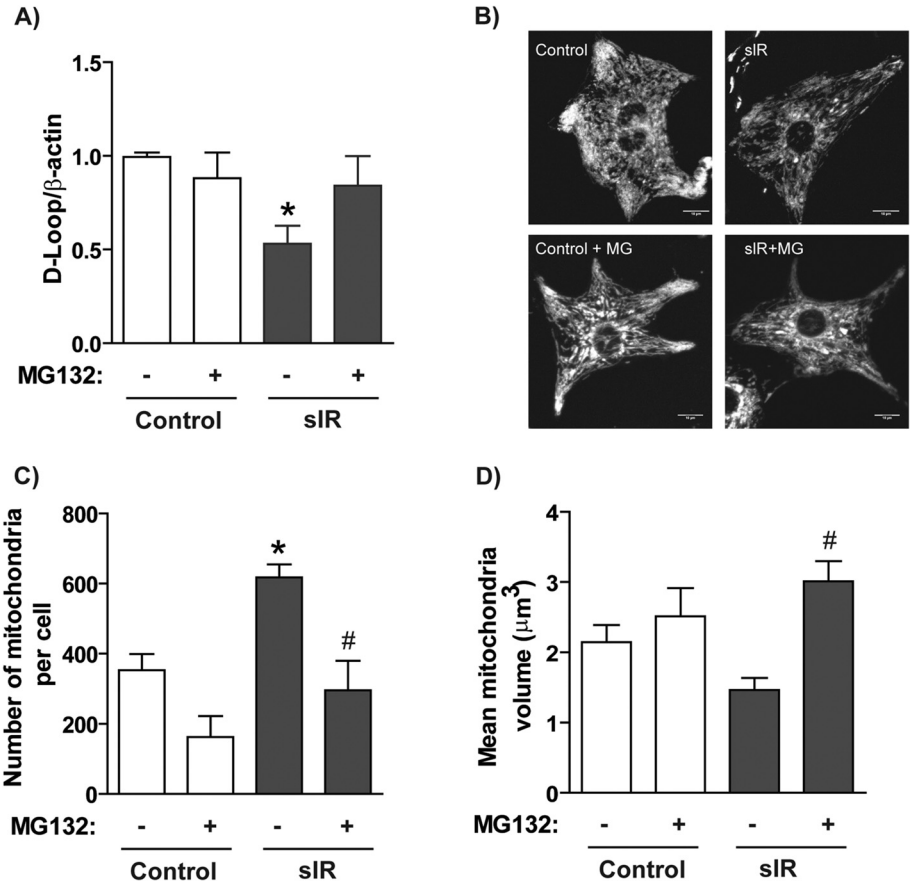

Fig. 3. Proteasome inhibition protects cardiomyocyte mitochondria from sIR. A) Mitochondrial DNA D-Loop region expressed as the D-Loop/β-actin ratio in control NRVMs and NRVMs after sIR, with or without MG132 (4 μM), (n = 5–7). B) Representative images of mitochondria stained with MitoTracker Green FM, used to evaluate: C) Number of mitochondria per cell and D) Mitochondrial volume (n = 4–6). Values are shown as mean ± SEM. *p < .05 *vs.* control and $^{\#}$*vs.* sIR.

dynamics, we induced a shorter period of ischemia (2 h) and reperfusion (1 h) in NRVMs in order to study mitochondrial changes during the early stages of the IR, before the cardiomyocyte injury was evident. Using confocal microscopy, we observed an increased number of mitochondria and reduced mitochondrial volume after sIR (Fig. 3B–D), indicating a fragmented phenotype. However, inhibition of the proteasome with MG132 prevented these sIR-induced changes in mitochondrial dynamics. Furthermore, increased reactive oxygen species (ROS) is a hallmark of IR, and mitochondria damage is a major sources of ROS [33]; therefore, we measured total ROS after a brief period of sIR. Our results demonstrated that MG132 prevents increased ROS produced during sIR in cardiomyocytes (Supplementary Fig. 1F). Taken together, our data indicate that proteasome inhibition prevents the mitochondrial fission and degradation induced by sIR in NRVMs.

3.5. Mitofusin-2 protects cardiomyocytes from IR injury

To study the role of Mfn2 in protection against cell death during IR, we overexpressed Mfn2 using an adenovirus to transduce NRVMs at two different MOI, 1 and 50 (Fig. 4A). We then measured cell death by quantifying the release of LDH. Our results showed that while Mfn2 overexpression at an MOI of 50 did not protect cardiomyocytes from death, with necrosis remaining near baseline levels, overexpression at a lower MOI (1) completely prevented sIR-induced necrosis (Fig. 4B). These results show that Mfn2 is directly involved in cardiomyocyte protection during IR, with an efficacy that depends on concentration. Moreover, NRVMs knocked down to Mfn2 showed increased LDH release in baseline conditions as compared to control, suggesting a crucial role for Mfn2 to maintain the cardiomyocytes survival. During sIR, NRVMs knocked down for Mfn2 did not show difference in the cell death as compared to the sIR condition (Fig. 4C).

To test whether these protective effects in cardiomyocytes are selective for Mfn2, we evaluated whether preservation of Miro1, another protein similar to Mfn2, might protect cardiomyocytes from sIR injury as effectively as Mfn2. Our data showed that Miro1 was expressed in the cardiomyocytes. As with Mfn2 (but not Mfn1), Miro1 protein levels decreased after sIR, an effect that was completely abolished by MG132 (Fig. 4D). To assess the role of Miro1 in protecting NRVMs from sIR, we overexpressed Miro1 with an adenovirus at MOIs of 10, 20, and 50 (Fig. 4E) and measured the release of LDH after sIR. Miro1 overexpression did not protect cardiomyocytes from sIR-induced cell death (Fig. 4F). These results suggest that Mfn2 has a unique and crucial role among mitochondrial proteins in cardioprotection during IR.

4. Discussion

The present work shows that inhibition of the proteasome confers cardioprotection against IR injury. The main findings were: a) proteasome inhibition prevents degradation of the mitochondrial fusion protein Mfn2 after sIR and preserves the mitochondria; b) Mfn2 degradation is an autophagy-independent process; and c) Mfn2 is essential for reducing sIR-induced cell death while Miro1 is not.

The proteasome is one of the main protein degradation systems in cells. Proteasome inhibition prevents degradation of various proteins during cardiac IR, reducing IR-induced cell death through different pathways [24,29]. However, the role of proteasome inhibition in cardioprotection is still controversial, and the effect seems to depend on the concentration of the inhibitor [34–40]. We first replicated our previously-reported results, in which 0.5 μM of MG132 decreased myocardial infarct size after *ex vivo* IR. However, 6 μM did not produce this protective effect [24]. On the other hand while neither, 2 nor 8 μM of MG132 affected sIR-induced necrosis, 4 μM of MG132 completely prevented this type of cell death. *Ex vivo* and *in vitro* models of IR are

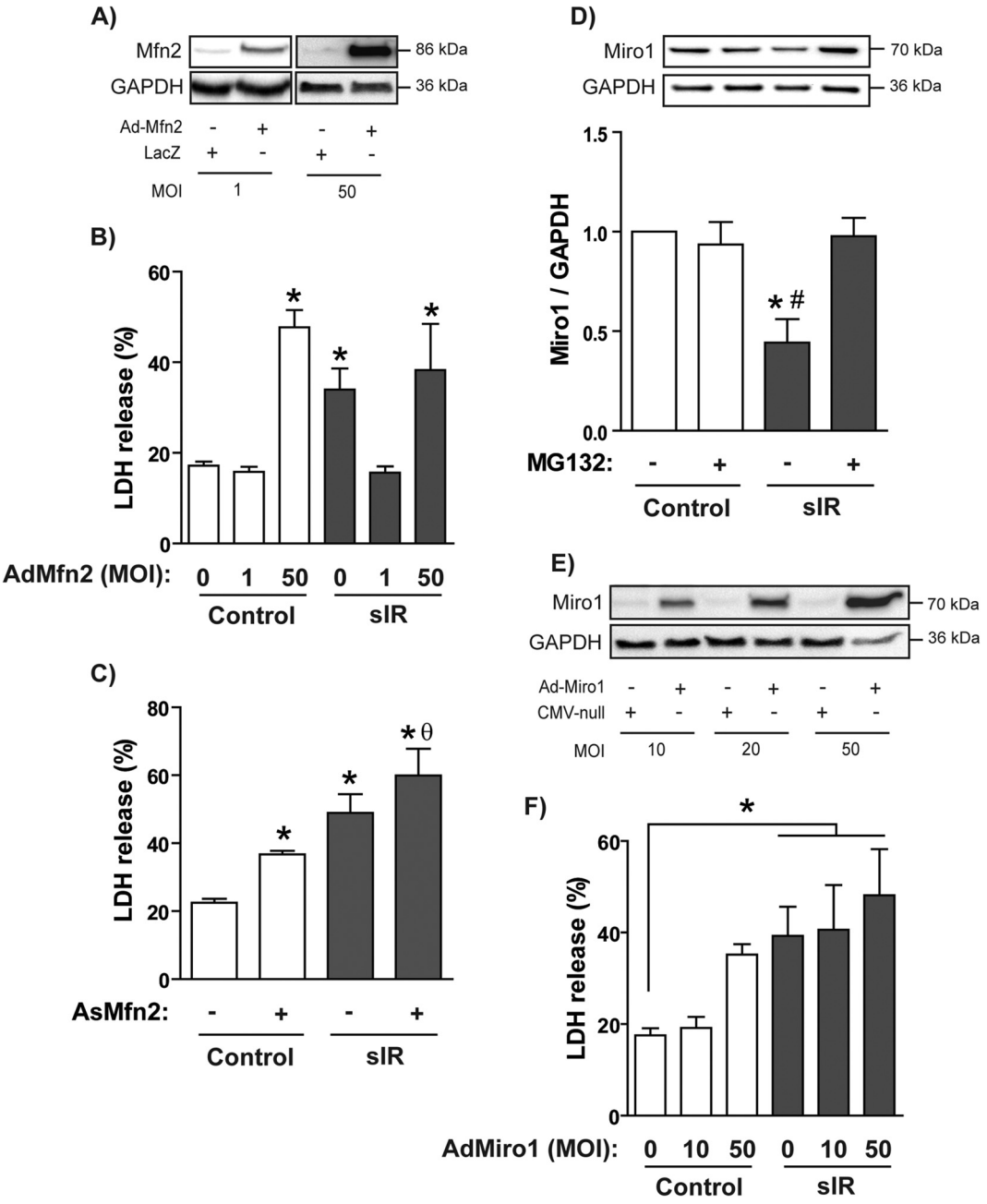

Fig. 4. Overexpression of Mfn2 protects cardiomyocytes from sIR injury. A) Representative Western blot showing Mfn2 overexpression in NRVMs at different MOIs (1 and 50). B) Percentage of lactate dehydrogenase (LDH) released into the culture medium of control NRVMs and NRVMs after sIR (n = 4–7). C) LDH release into the culture medium in NRVMs knocked down for Mfn2 (AsMfn2), $n = 4$. D) Western blot of Miro1 and GAPDH (loading control) and bar graph of Miro1 protein content (n = 4–5). E) Representative Western blot showing Miro1 overexpression in NRVMs at different MOIs (10, 20 and 50). F) LDH released into the culture medium (%) from NRVMs with overexpressed Miro1 (MOIs: 1 and 50) in controls and after sIR (5–8). *p < .05 *vs.* control, $^{\#}$*vs.* sIR + MG132 and $^{\theta}$vs. AsMfn2.

complementary but different; for example, neonatal cells are more resistant to ischemia than adult cardiomyocytes [41]. Therefore, we used different IR protocols in the two models (in terms of time and inhibitor concentration). Differences in the dose-dependent protective effects of MG132 could be attributable to the higher concentration of MG132 required for the *ex vivo* model, which may have inhibited all proteasome activity and/or other proteases or pathways, inducing an increase in cell death, as we showed in a previous report for ixazomib [24].

Moreover, dose-dependent inhibition of CT-like activity with MG132 may be related to the NRVM protection during sIR and therefore, 2 μM could be insufficient to prevent the damage. On the other hand, 8 μM of MG132 inhibited both CT-like and T-like activities. However it did not prevent the NRVM damage induced by sIR, suggesting that simultaneous inhibition of both activities may be detrimental and contribute to the loss of the protective effect of MG132. Further experiments will be necessary to explore this possibility.

Mitochondria are dynamic organelles that undergo various changes in size, shape, and subcellular localization [42,43]. During IR, fission becomes necessary after mitochondrial damage to degrade dysfunctional mitochondria and minimize cell injury. In this context, fission protects the cell by allowing for autophagic degradation of damaged mitochondria. However, excessive fission leads to mitochondrial mass loss, ATP deficits, apoptosis activation, and cardiomyocyte death. Unregulated mitochondrial degradation, then, represents a common pathway for IR-induced cardiomyocyte damage [44–48]. Therefore, it has been suggested that preserving the mitochondria from degradation

in the context of IR injury could protect the myocardium from infarction [13,45,49,50]. Mfn2 is a mitochondrial protein, crucial for promoting and maintaining mitochondrial fusion and metabolic function [43,51]. It has been reported that Mfn2 is degraded through the proteasome under both baseline and stress conditions in cells other than cardiomyocytes [25–27,52]. Nonetheless, the mechanisms mediating Mfn2 degradation in the setting of IR injury, and the role of Mfn2 role in cardioprotection, remain controversial. Our study shows that Mfn2 is degraded *via* the proteasome at baseline and during sIR, suggesting that Mfn2 degradation may be one of mechanisms underlying myocardial damage after IR. We showed that MG132 prevents Mfn2 degradation both *ex vivo* and *in vitro*. During sIR, 2 or 8 μM of MG132 only partially prevented degradation of Mfn2, while a 4 μM dose completely abolished this degradation. This effect confers protection against cardiomyocyte death, as measured by LDH release during sIR. Moreover, previous reports suggested that preserved levels of Mfn2 attenuate ROS production and cardiomyocyte injury induced by doxorubicin or angiotensin II [53,54]. Our results show that sIR-induced increased ROS levels can be prevented by MG132.

Cardiomyocytes subjected to 2 h of ischemia and 1 h of reperfusion exhibited mitochondrial fission. Furthermore, after 6 h of ischemia and 16 h of reperfusion, we observed a decrease in mitochondrial content, possibly as a consequence of sustained mitochondrial fission. This finding suggests that preventing Mfn2 degradation may preserve the mitochondria and confer cardioprotection.

Indeed, NRVMs knocked down for Mfn2 expression with an antisense adenovirus increased cell death at baseline condition, thereby relating Mfn2 protein levels and cardiomyocytes viability. However, knocked down for Mfn2 did not increase the damage induced by sIR, probably because the antisense adenovirus against Mfn2 does not decrease the levels of Mfn2 below the ones observed during sIR condition. Importantly, the efficiency of the antisense adenovirus against Mfn2 has been described previously [55]. Moreover, Mfn2 overexpression seems to protect cardiomyocytes from sIR-induced death; however, this effect seems to depend heavily on Mfn2 concentration, as the highest levels of overexpression tested did not prevent IR-induced injury. These results could explain, at least in part, the controversial reports on the role of Mfn2 in cardioprotection and IR. Elevated Mfn2 overexpression may waste needed energy and/or induce an endoplasmic reticulum stress that is harmful for the cell during IR or even at baseline. On the other hand, Mfn2 is involved in mitochondria/sarcoplasmic reticulum tethering, which causes mitochondrial calcium overload, ROS production, and mitochondrial dysfunction during IR injury [16]. Therefore, overexpression using an adenovirus at an MOI 50 may produce excessive levels of the Mfn2 protein that maintains mitochondria/sarcoplasmic reticulum tethering, provoking mitochondrial dysfunction and cardiomyocyte death. Additional experiments will be necessary to study these hypotheses.

Other proteins are also involved in regulating mitochondrial dynamics and mitophagy. Mfn1 and Miro1 have been reported to interact with Mfn2. Similar to Mfn2, both are involved in regulating mitochondrial morphology (inducing mitochondrial fusion) and turnover [56]. Miro1 also regulates anterograde and retrograde mitochondrial motility in neuronal cells [9]. Mfn1 and Miro1 are known to be degraded by the proteasome in cell types other than cardiomyocytes [56–61]. To compare the consequences of proteasome inhibition on Mfn2 and two different mitochondrial proteins, we measured Mfn1 and Miro1 levels during sIR. Our data showed that Mfn1 protein levels did not change during IR or sIR, nor did levels change in the presence of the proteasomal inhibitor MG132. Although it has been described that Mfn1 levels decrease during hypoxia/reoxygenation [62], different sIR protocols could explain these controversial data. On the other hand, mitochondrial fission can occur asymmetrically, and some mitochondrial components are preserved over others [63,64]. Finally, our results suggest that Mfn2 seems to be preferentially degraded by the proteasomal route in cardiomyocytes since MG132 increases Mfn2 but not Mfn1 content under control conditions. Future studies are necessary to confirm these questions.

Our findings show that Miro1 was expressed in cardiomyocytes, and like Mfn2, its abundance was reduced in NRVMs subjected to sIR. Moreover, inhibition of the proteasome also prevented Miro1 degradation, as observed for Mfn2. Nonetheless, overexpression of Mfn2 but not Miro1 conferred protection against reperfusion-induced necrosis, highlighting a role for Mfn2 as a therapeutic target for attenuating IR injury. This comparison with another protein involved in mitochondrial dynamics further strengthens the hypothesis that Mfn2 may have a key and specific role in cardioprotection. Since all of these data were obtained from homogenized tissues, and these proteins are expressed in both the cytoplasm and mitochondria, it would be helpful to corroborate these changes in mitochondrial fractions.

In addition to the proteasome system, autophagy is another biological process responsible for degradation of proteins and organelles in the cell [65]. A link between these two pathways has been suggested. Autophagy may be upregulated upon proteasome inhibition, and both protein degradation systems have common substrates and regulatory components [32]. It has been thoroughly described that autophagy is activated during IR injury, with mitochondria degraded by mitophagy [66]. However, the role of autophagy in cardioprotection remains controversial [67–72]. In our study, Mfn2 could be degraded by a pathway other than the proteasome during IR, as MG132 did not completely prevent degradation of this protein. Therefore, we evaluated a potential compensatory role for autophagy in Mfn2 degradation in our sIR model. sIR induced an increase in LC3II protein content, independent of the presence or absence of MG132. Additionally, our autophagic flux experiments further confirmed this finding, suggesting that autophagy activation after sIR occurs in a proteasome-independent manner. We also observed that inhibition of autophagy during sIR in the presence of MG132 impaired the protective effect of proteasome inhibition on Mfn2 degradation. Nonetheless, administration of BA in the absence of MG132 did not prevent Mfn2 loss, suggesting that degradation of this protein does not depend on autophagy. Interestingly, however, BA did not alter the inhibitory effects of MG132 on the chymotrypsin-like activity of the proteasome, which may imply that simultaneous inhibition of both degradation systems may promote activation of an alternative degradation mechanism, such as another protease pathway. Furthermore, BA inhibited the trypsin-like activity of the proteasome, but this effect was not associated with decreased Mfn2 degradation, suggesting that chymotrypsin-like activity is responsible for preventing the loss of this protein in IR. Further investigation is necessary to confirm this hypothesis.

In conclusion, our results show that the proteasome is the principal system responsible for Mfn2 degradation during IR in cardiomyocytes. Moreover, our data suggest that even after autophagy is activated during sIR, the mitochondria are protected when the proteasome is inhibited at cardioprotective levels. This effect may be related to inhibition of Mfn2 degradation. Therefore, preserving specific levels of Mfn2 by inhibiting the proteasome may be a valuable cardioprotective strategy. A schematic representation of the proposed mechanism is shown in Fig. 5.

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Transparency document

The Transparency document associated with this article can be found, in online version.

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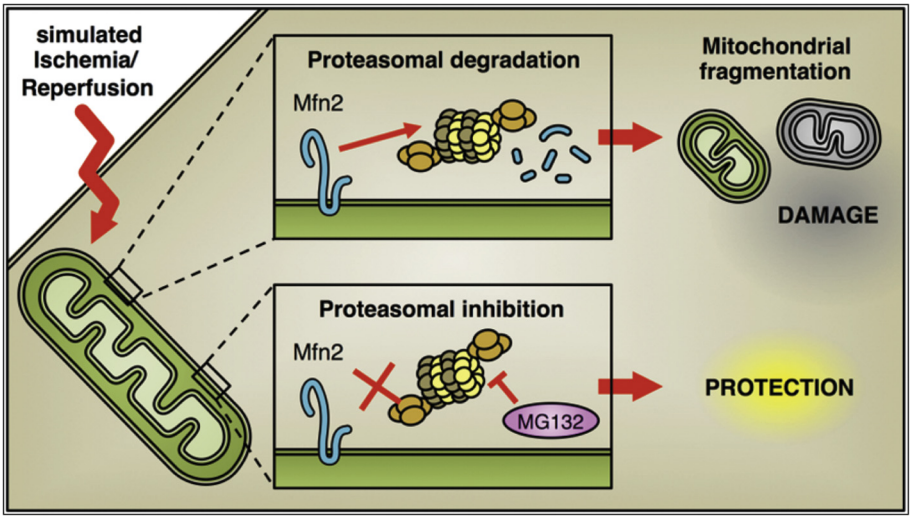

Fig. 5. Model representing the proposed cardioprotection route induced by proteasome inhibition.

1130407 to G.S, 1180613 to Z.P., 11170962 to I.O., 1160704 to P.D., 1200490 to S.L., 11181000 to J.A.R., 3190546 to C.L C., and 3160549 to P.A.; and Fondo de Financiamiento de Centros de Investigación en Áreas Prioritarias (FONDAP) grant 15130011 to S.L., J.A.R., and Z.P. We thank Dr. A. Zorzano that kindly provided the adenovirus AsMfn2.

Author contributions

IO, GP, JR, PA, MCD, and CL-C performed most of the experiments and data analysis. ZP, SL, PD, and GS conceived the project. ZP designed the experiments and supervised the research. ZP, GS, and SL wrote the manuscript. All the authors reviewed and accepted the manuscript.

Declaration of competing interest

The authors declare that they have no competing interests; financial or otherwise.

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