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Pharmacological inhibition of translocon is sufficient to alleviate endoplasmic reticulum stress and improve Ca^{2+} handling and contractile recovery of stunned myocardium.

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ABSTRACT

Introduction: The function of endoplasmic reticulum (ER), a Ca^{2+} storage compartment and site of protein folding, is altered by disruption of intracellular homeostasis. Misfolded proteins accumulated in the ER lead to ER stress (ERS), unfolded protein response (UPR) activation and ER Ca^{2+} loss. Myocardial stunning is a temporary contractile dysfunction, which occurs after brief ischemic periods with minimal or no cell death, being oxidative stress and Ca^{2+} overload potential underlying mechanisms. Myocardial stunning induces ERS response with negatively impact on the post-ischemic mechanical performance through an unknown mechanism.

Aims: In this study, we explored whether ER Ca^{2+} efflux through the translocon, a major Ca^{2+} leak channel, contributes to Ca^{2+} mishandling and the consequent contractile abnormalities of the stunned myocardium.

Methods: Mechanical performance, cytosolic Ca^{2+}, UPR markers and oxidative state were evaluated in perfused rat/mouse hearts subjected to a brief ischemia followed by reperfusion (I/R) in absence or presence of the translocon inhibitor, emetine (1 μM), comparing its effects with those of the chaperones TUDCA (30 μM) and 4-PBA (3 mM).

Results: Emetine treatment precluded the I/R-induced increase in UPR signaling markers and improved the contractile recovery together with a remarkable attenuation in myocardial stiffness when compared to I/R hearts with no drug. This alleviation of I/R-induced mechanical abnormalities was more effective than that obtained with the chemical chaperones, TUDCA and 4-PBA. Moreover, emetine treatment produced a striking improvement in diastolic Ca^{2+} handling with a partial recovery of the I/R-induced oxidative stress.

Conclusion: Blocking ER Ca^{2+} store depletion via translocon suppressed ER stress and improved mechanical performance and diastolic Ca^{2+} handling of stunned myocardium. Modulation of

translocon permeability emerges as a therapeutic approach to face dysfunctional consequences of the I/R injury.

KEYWORDS

MYOCARDIAL ISCHEMIA/REPERFUSION – ENDOPLASMIC RETICULUM STRESS - TRANSLOCON

1. INTRODUCTION

Myocardial stunning is a temporary post-ischemic contractile dysfunction, which occurs despite the re-establishment of normal coronary flow after a brief ischemia and in the absence of cell death[1,2]. It occurs in different clinical settings[3] and multiple studies have identified an increase in oxygen-derived free radicals and a Ca^{2+} overload, as potential triggers of this phenomenon[4]. Both intracellular alterations perturb the function of the endoplasmic reticulum (ER)[5]. This membrane-bound organelle is an intracellular Ca^{2+} store involved in folding and sorting of newly synthesized secretory and membrane proteins. Moreover, cardiomyocytes contain the sarcoplasmic reticulum (SR), a differentiated form of ER highly specialized in the regulation of Ca^{2+} cycling that drives contraction and relaxation[6]. Whether ER and SR are two physically separated membrane networks or a unique system with different specialization is still under debate[7].

When ER protein folding capacity is disrupted, unfolded proteins accumulate in the ER, a state known as ER stress (ERS), triggering the unfolded protein response (UPR)[8]. The UPR is initiated by the activation of three sensors: activating transcription factor 6 (ATF6), PKR-like ER kinase (PERK), and inositol-requirement enzyme 1 α (IRE1α) and promotes up-regulation of ER chaperones, such as GRP78, attenuates protein translation and accelerates ER-associated protein degradation (ERAD) and autophagy to eliminate unwanted proteins. However, during prolonged or overwhelming protein-folding stress, UPR switches the cytoprotective pathways into cell death programs.

Accumulated evidence has shown that in hearts submitted to a severe I/R insult the ERS response on cell survival is detrimental, aggravating I/R injury[9]. However, little was known about the ERS after a mild I/R insult. We recently demonstrated that ERS has also negative consequences in the stunned myocardium[10]. Inhibition of ERS enhanced the post-ischemic contractile recovery without

precluding the I/R-induced oxidative damage, suggesting that prevention of Ca^{2+} mishandling is a putative mechanism involved in this recovery.

The translocon, a protein complex of ER membranes involved in the transport of nascent peptides into the ER lumen, could function as a Ca^{2+} leak channel[11]. In the absence of translation, GRP78 seals the pore, but during peptide translocation, its opening allows for Ca^{2+} efflux. Moreover, when GRP78 is recruited by accumulated unfolded proteins, Ca^{2+} leakage exacerbates, intensifying ERS. Studies in non-myocytic cells demonstrated that different ERS inducers promote ER Ca^{2+} unloading and apoptosis, and both effects were efficiently prevented by translocon inhibitors[12,13].

The role of the translocon in the myocardium has been poorly studied. A recent report showed that a moderate ER Ca^{2+} depletion, produced by the translocon opener puromycin prior to ischemia, reduced the infarct size of mouse hearts subjected to an *in vivo* I/R protocol[14].

To date, the impact of the translocon in the stunned myocardium is unknown. We hypothesized that blockage of the translocon-mediated Ca^{2+} leak by limiting the cytosolic Ca^{2+} overload can mitigate the post-ischemic contractile dysfunction of the stunned myocardium. To address this hypothesis, we examined the impact and effectiveness of the translocon inhibitor, emetine, on the intensity of ERS, the cytosolic Ca^{2+}, the oxidative state and the post-ischemic performance of the stunned myocardium.

2. MATERIAL AND METHODS

2.1. Animals

Experiments were performed in male Wistar rats (200-300 g body weight) and in male C57/Bl6 mice (20-25 g body weight). Rat hearts were used for mechanical and biochemical determinations and cytosolic Ca^{2+} measurements were performed in mouse hearts to reduce costs in fluorescent indicator. Animals were inbreeded and maintained in our animal facilities in accordance with the Guide for the Care and Use of Laboratory Animals (NIH, 2011). The protocols were approved by the Institutional Animal Care and Use Committee (CICUAL) of School of Medicine, National University of La Plata, Argentina (Nro T05022014).

2.2. Ex vivo experiments: intact hearts

During this study, rats were anaesthetized with Ketamine/Xylazine (75 mg/kg/5 mg/kg) and heparinized with 250 U/Kg sodium heparin by intraperitoneal injection. The anesthetic dose was sufficient to produce a surgical level of anesthesia (loss of pedal withdrawal reflex) without profoundly affecting the cardiovascular function. Central thoracotomy and heart excision were performed immediately after phase III of anesthesia was reached.

Isolated hearts were perfused at constant temperature (37°C), heart rate (4Hz) and flow (14 ml/min) with physiological bicarbonate buffer solution (BBS) composed by (in mM): 128.3 NaCl, 4.7 KCl, 1.35 $CaCl_2$, 20.2 $NaHCO_3$, 0.4 NaH_2PO_4, 1.1 $MgSO_4$, 11.1 glucose and 0.04 Na_2EDTA. This solution was equilibrated with 95% O_2-5% CO_2 to give a pH of 7.4, as described[15]. The mechanical activity of the heart was assessed by introducing into the left ventricle (LV) a balloon connected to a pressure transducer (ADInstruments MLT 0380, CO, USA) and filled with aqueous solution to achieve a left ventricular end-diastolic pressure (LVEDP) of approximately 5-10 mmHg. LV contractility was evaluated measuring the developed-pressure (LVDP) and the maximal rate of

pressure development (+dP/dt). Relaxation was assessed by the time constant of left ventricular developed-pressure decay (Tau), and total relaxation time (TR). Tau was obtained by fitting the time course of LVDP fall with a monoexponential function assuming zero pressure asymptote. Experimental fitting was performed from the time of the maximal rate of pressure decline (-dP/dt) to a level of 5 mmHg above the LVEDP. TR was the time of the pressure decline from the peak of LVDP to the LVEDP. LVDP, +dP/dt, LVEDP and TR were expressed in absolute values and Tau was expressed as differences from pre-ischemic values.

When mouse hearts were used, central thoracotomy and heart excision were performed after cervical dislocation. Langendorff perfusion of the isolated mouse hearts was modified as follows: heart rate 5 Hz, constant perfusion pressure of 80 mmHg, at 34°C with Tyrode solution containing (in mM): 140 NaCl, 5.4 KCl, 2 $CaCl_2$, 0.33 KH_2PO_4, 1 $MgCl_2$, 10 glucose, and 10 HEPES; pH 7.4; equilibrated with 100% O_2[16].

2.3. Experimental protocols

After a 10-min stabilization period, rat hearts were subjected to 20 min of global normothermic ischemia followed by 30 min of reperfusion. We have previously demonstrated that this I/R protocol produced neither the increase in LDH release, as a readout of cell necrosis, nor the enhancement of TUNEL positive nuclei cells, indicative of apoptosis[10]. Moreover, no increase in the ER stress pro-apoptic marker, CHOP, was found[10]. The I/R protocol was performed in the absence (I/R) or the presence of 1 μM emetine (I/R + eme). Emetine was administered 5 min prior to ischemia and it was present during the ischemic period and in the first min of reperfusion. Chemical chaperones, 3 mM 4-phenylbutyrate (4-PBA) and 30 μM tauroursodeoxycholic acid (TUDCA) were perfused during the reperfusion period as previously described[10]. Non-ischemic hearts perfused during times to match the duration of I/R protocol, were used as controls. At the

end of the protocols, hearts were freeze-clamped and stored at -80°C for biochemical determinations.

2.4. Measurement of intracellular Ca^{2+}

To measure intracellular free [Ca^{2+}], mouse hearts were loaded with 30 μM of the acetoxymethyl ester form (AM) of the Ca^{2+}-sensitive fluorescent indicator Rhod-2 (Invitrogen) as previously described, using a custom-made setup for pulse local-field fluorescence (PLFF) microscopy[17,18]. The level of loading during incubation was examined every 5 min by measuring the basal fluorescence. To wash out the dye hearts were perfused with fluorophore-free normal Tyrode for 5-10 min, before starting fluorescence recordings. The recordings were obtained at near physiological temperature by gently placing one end of the optic fiber on the tissue used for dye excitation (532 nm laser light). Blebbistatin (10 μM) was used to minimize movement artifacts. Emitted light from the loaded fluorophore was carried back through the same fiber optic, filtered with a 590 nm Longpass Glass Color Filter (Edmund Scientific USA) and focused on an avalanche photodiode (EG & G, Canada) connected to a PowerLab410 (ADinstruments). Diastolic Ca^{2+} is expressed compared to pre-ischemic values, expressed as ratio between emitted (F–F0) and basal (F0) fluorescence (ΔF/F0). Although our loading conditions would favor a prominent cytosolic localization of Rhod-2, we cannot exclude that a proportion of total fluorescence arises from within mitochondria[19]. After stabilization, hearts were subjected to 15 min of normothermic global ischemia, followed by 10 min of reperfusion. This protocol was chosen based on the results of previous experiments, which indicate that this ischemic period produced a reversible altered Ca^{2+} handling and mechanical dysfunction, typical of the stunned heart[16].

2.5. Electrophoresis and Western Blot

Pulverized frozen ventricles were homogenized using a handheld homogenizer (Kinematica Polytron 1200 PT E) in 5 vol. of the following: 20 mM Tris-HCl, 5 mM EDTA, 2.5 mM EGTA, 10 mM NaCl, 300 mM sucrose, 1% IGEPAL, 0,1% SDS, 25 mM NaF, 1 mM PMSF, 1 mM Benzamidine, 10 μg/ml Leupeptin, 1 μg/ml pepstatin, 1 μg/ml E64 [trans-Epoxysuccinyl-L-leucylamido(4-guanidino)butane]. The homogenate was sedimented at 13000 x g for 10 min and protein concentration was measured using the Bradford method with bovine serum albumin (BSA) as the standard in the supernatant. 50 μg of protein was electrophoresed per gel lane in 12% acrylamide gels according to Laemmli[20]. Proteins were transferred to PVDF membranes (Immobilion-P, Millipore). Blots were blocked with Tris-buffered saline (50 mM Tris, 150 mM NaCl, pH 7.4) with the addition of 0.1% Tween (TBST) and 5% skim milk or BSA, as indicated for each antibody, and then incubated overnight with the primary antibodies: phospho-eIF2α (Cell Signaling Technology, 1:1000 in 5% BSA TBST), eIF2α (Invitrogen, 1:1000 in 1% milk TBST) and GAPDH (Millipore, 1:10000 in 1% milk TBST). Membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, 1:10000 in 1% milk TBST) and developed using an enhanced chemiluminescence reagent (Millipore). Chemiluminescence signals were detected using Chemidoc Imaging system (Bio-Rad) and analyzed with ImageJ software (NIH).

2.6. Real time qPCR

RNA was extracted from hearts using TRIzol reagent (Life Technologies, Carlsbad, Calif). cDNA was generated by reverse transcriptase reaction using M-MLV RT (Promega, Madison, Wis). Real time quantitative PCR was performed on cDNA using the SYBR Select Master Mix (Life Technologies, Carlsbad, Calif) and iCycleriQ (Bio-Rad, Hercules, Calif). The following primers were used:

Gene	Sense (5' to 3')	Antisense (5' to 3')
GRP78	TGATTCCGAGGAACACTGTGG	GGTGATTGTCTTTTGTCAGGGG
Spliced XBP1	GAGTCCGCAGCAGGTGC	GGTCCAACTTGTCCAGAATGC
GAPDH	GGGTGTGAACCACGAGAAAT	CCACAGTCTTCTGAGTGGCA

Relative abundance of RNA was calculated by the ΔΔCt method[21]. Primers were designed using Primer-Blast (NCBI, NIH). All primers were between 90% and 110% efficient, as assessed by standard curve, and all displayed only 1 dissociation peak.

2.7. Assessment of reduced GSH Levels

Total reduced GSH content was determined according to a modified Ellman's method[22]. Briefly, powered ventricular tissue was homogenized in 5 vol. of a buffer solution containing 140 mM KCl and 20 mM KH_2PO_4 (pH 7 at 4°C) and then centrifuged at 1000 x g for 10 min. The supernatant was treated with 28 % tricarboxylic acid and then sedimented at 5500 x g for 10 min. GSH levels in the supernatant were measured based on the reaction of non-protein sulfhydryl groups with 5,5'dithiobis 2-nitrobenzoic acid (0.01 M) in 0.4 M Tris buffer pH 8.9 to give a compound that absorbs at 414 nm. GSH content was expressed as µg per mg of protein measured in the homogenate by the Bradford method.

2.8. Statistics

Data are expressed as mean ± SEM. Statistical significance was determined by Student's test for paired or unpaired observations as appropriate, and ANOVA when different groups were compared. The Newman-Keuls test was used to examine statistical differences observed with the ANOVA. A P value <0.05 was considered statistically significant.

3. RESULTS

3.1. Inhibition of the translocon prevents stunning-induced UPR triggering

In order to explore if the pharmacological inhibition of the translocon complex prevents the I/R-induced activation of the UPR, we evaluated the mRNA expression of sXBP1, a transcription factor generated via the endoribonuclease activity of IRE1α and GRP78, the major ER chaperone. Furthermore, we assessed the phosphorylation of eIF2α, associated to a rapid halt in protein translation, an early event in the ER stress response. Rat hearts were subjected to 20 min of ischemia followed by 30 min of reperfusion in the presence or the absence of the translocon inhibitor, emetine. Compared to non-ischemic controls, I/R hearts showed the expected ER stress response as demonstrated by the increase in sXBP1 and GRP78 mRNA levels (Figure 1a and b) and eIF2α phosphorylation (Figure 1c). The enhancement of all three UPR markers was suppressed by emetine treatment. Thus, inhibition of the translocon seems to be enough to preclude the I/R-induced ER stress.

3.2. Emetine improves the post-ischemic mechanical recovery of the stunned myocardium

We next studied the effects of emetine on the post-ischemic mechanical recovery. Figure 2a and b shows the time course of contractility indexes, LVDP and +dP/dt, of hearts submitted to I/R in the absence or the presence of 1 μM emetine. The contractile recovery after the ischemic period was significantly higher in hearts treated with emetine than in non-treated hearts. At 30 min of reperfusion LVDP and +dP/dt attained 107.0 ± 8.8% and 112.7 ± 12.9% of pre-ischemic values in the presence of emetine, while the recovery of these parameters was 60.6 ± 10.4% and 45.2 ± 5.0% respectively, in the absence of the drug. The emetine-induced improvement in contractile recovery was associated with a significant decrease in the contracture developed during ischemia

and reperfusion (Figure 2c). In non-treated hearts, LVEDP, an index of myocardial stiffness, increased towards the end of the ischemic period and even further upon reperfusion, an elevation that persisted during the whole reperfusion period. Emetine drastically reduced the enhancement of LVEDP. Finally, Figure 2d shows the time course of Tau, a relaxation parameter of the same hearts. At the onset of reperfusion, a prolongation of Tau was observed in both treated and untreated groups (antirelaxant effect) which then tended to return to pre-ischemic values. Emetine accelerated the recovery of Tau during reperfusion. This beneficial effect on relaxation was also reflected by another parameter, the total relaxation time (RT). At the end of reperfusion, RT was 110.5 ± 3.4 msec (n=7) in the non-treated group and significantly decreased to 84.4 ± 2.6 msec (n=4) in the presence of emetine. Overall, the results indicate that inhibition of the translocon improved the post-ischemic recovery of cardiac mechanical function. The cardioprotection provided by emetine was associated to the amelioration of ER stress.

3.3. Comparison of the effects of emetine and chemical chaperones on the mechanical dysfunction of the stunned myocardium

Since the chemical chaperones, TUDCA and 4-PBA, also helped to mitigate the post-ischemic mechanical dysfunction of stunned hearts[10], we next compared their beneficial effects with those observed by the translocon inhibitor. Figure 3 shows the mechanical parameters at the end of the reperfusion period of hearts subjected to I/R in the absence or the presence of the three ER stress relievers. We found that emetine treatment produced a better recovery in contractile parameters than the chemical chaperones, reaching statistical significance in LVDP (Figure 3a and b). Moreover, the marked emetine-induced reduction of the post-ischemic contracture (LVEDP) was not mimic by the chemical chaperones (Figure 3c). Finally, emetine as TUDCA significantly improved myocardial relaxation after I/R, while 4-PBA showed a tendency to diminish the time constant of relaxation Tau when compared to non-treated hearts (Figure 3d). Overall, the data

demonstrate that emetine worked better than TUDCA and 4-PBA in alleviating the I/R-induced abnormalities in LVDP and LVEDP in perfused rat hearts.

3.4. Translocon blockage partially attenuates the I/R-induced oxidative stress

It is well known that oxidative stress is a contributing factor to myocardial stunning[4] and a promoter of ER stress[23]. We therefore explored if emetine effects on UPR activation and contractile recovery were due to alleviation of the I/R-induced oxidative stress. The total content of the reduced form of glutathione (GSH), a main cytosolic antioxidant defense, was measured as a marker of oxidative stress. As expected, I/R leads to a decrease in GSH levels compared with the non-ischemic hearts and the presence of emetine partially reversed this reduction (Figure 4). The results indicate that the protective effects of emetine on ER stress and contractile post-ischemic dysfunction cannot be fully explained by the recovery of the redox environment.

3.5. Emetine prevents the I/R-induced increased in diastolic Ca^{2+}

Finally, we examined the contribution of the translocon-mediated ER Ca^{2+} leakage to the disruption of cytosolic Ca^{2+} homeostasis occurring during I/R. Mouse hearts were loaded with a fluorescent Ca^{2+} indicator and subjected to an I/R stunning protocol in the absence or the presence of the translocon inhibitor.

Figure 5a shows Ca^{2+} transients at critical time points throughout the I/R. The cytosolic Ca^{2+} overload occurring at the first min of reperfusion, was due to an enhancement in diastolic Ca^{2+} without significant changes in the amplitude of the Ca^{2+} transient. Emetine blunted this increase. Figure 5b shows the overall results of the time course of diastolic Ca^{2+} during I/R. In the absence of emetine, diastolic Ca^{2+} increased during ischemia, reached maximum levels at the onset of reperfusion and then decreased towards pre-ischemic values. In the presence of emetine, neither

the ischemic diastolic Ca^{2+} increase nor the reperfusion-induced Ca^{2+} bump were observed. Interestingly, these changes paralleled those induced by I/R in LVEDP (Figure 2c).

The data indicate that inhibition of the translocon-mediated ER Ca^{2+} depletion has a major effect on the prevention of Ca^{2+} mishandling during I/R, which would explain the lack of stunned-induced enhancement in myocardial stiffness.

4. DISCUSSION

Reperfusion after brief periods of ischemia causes a myocardial reversible contractile dysfunction with minimal or no cell death known as stunned heart[1,2]. This phenomenon is important in different clinical settings such as the spontaneous reperfusion after coronary spasm or post-exercise ischemia, after coronary reperfusion by thrombolytic therapy, percutaneous coronary intervention or primary angioplasty, or after cardioplegic arrest for cardiac bypass surgery[3]. In this scenario ER function can also be disturbed, resulting in accumulation of misfolded proteins and ER stress. In a previous study, we demonstrated that the ER stress and the UPR signaling were triggered in the stunned myocardium[10]. Due to the poor severity of the stressor and the lack of cell death in this situation, an adaptive response of the UPR signaling was expected. However, it was the prevention of ER stress by the chemical chaperones 4-PBA and TUDCA, which turned out to be beneficial for the post-ischemic mechanical recovery of this mild I/R injury. The fact that the contractile improvement occurred without mitigation of the I/R-induced oxidative stress suggested that the chemical chaperones were alleviating the intracellular Ca^{2+} mishandling occurring upon reperfusion, the other potential culprit in the pathogenesis of myocardial stunning. The present study represents an important step forward establishing that the Ca^{+2} efflux through the translocon, a component of the ER translation machinery and a major Ca^{2+} leak channel[24,25], is critical in determining the ER stress, diastolic Ca^{2+} mishandling and mechanical abnormalities in the stunned myocardium.

The role of the translocon was studied by the use of emetine. At the molecular level, this emetic alkaloid present in ipecac syrup interacts with the small ribosomal subunit preventing the detachment of the nascent polypeptide/ribosome complex from the translocon[26]. In this way, emetine not only inhibits protein synthesis but also blocks the translocon and therefore the putative ER Ca^{2+} leakage. In various cell types, emetine was found to decrease ER Ca^{2+} leak induced

by diverse ER stressors: tunicamycin in Xenopus oocytes[12] thapsigargin in human salivary gland cells [27] and puromycin in adult mouse cardiomyocytes[14]. Moreover, in Xenopus oocytes Paredes at al. demonstrated that 1 μM of the translocon inhibitor was effective in blocking ER stress[12]. However, little is known about the effect of emetine against a pathophysiological ER stress inducer, such as the I/R injury. Our experiments showed that in stunned hearts emetine decreased three important UPR signaling markers and prevented the enhancement in diastolic Ca^{2+}, suggesting that there is an ER Ca^{2+} leakage through the translocon which is crucial in the progress of ER stress during myocardial stunning.

Studies from Valverde et al. using the same stunned-heart model, demonstrated that at the onset of reperfusion, there is a depletion of SR Ca^{2+} content associated to a marked increase in diastolic Ca^{2+} [18]. Their experiments led them to propose that SR Ca^{2+} loss was contributing to the cytosolic Ca^{2+} overload at the onset of reflow. The current results unexpectedly bring the ER into the scene of the critical phase of the first minutes of reperfusion, demonstrating that Ca^{2+} leak via the translocon is also a mechanism contributing to the disruption of Ca^{2+} homeostasis during this time.

In a recent work, Al Mawla et al. studied the role of the translocon in the irreversible I/R injury[14]. Their results indicated ER Ca^{2+} depletion by puromycin-mediated activation of the translocon prior to ischemia, was cardioprotective in mice subjected to *in vivo* I/R. These findings are in line with our results since both, reducing ER Ca^{2+} content by a translocon opener before ischemia as well as preventing ER Ca^{2+} leak by a translocon inhibitor during I/R, converge in the attenuation of the cytosolic Ca^{2+} overload at the onset of reperfusion, with favorable consequences on the cardiac outcome: cell death alleviation in the irreversible I/R injury[14] and prevention of impaired contractile performance in the reversible I/R injury (present results).

We cannot rule out, however, that the cardioprotection produced by emetine was in part due to the decrease in the I/R-induced oxidative stress. It is well known that the ER/SR interacts and

affects mitochondrial function[28]. The transfer of Ca^{2+} from the ER/SR to the mitochondria plays a key role in supporting ATP production. However, excessive mitochondrial Ca^{2+} load, as occurs during I/R, leads to overactivation of electron transport chain and enhanced ROS production[29]. It is possible that, by precluding ER Ca^{2+} leakage and alleviating diastolic Ca^{2+} overload, emetine could be reducing mitochondrial Ca^{2+} accumulation and thus, limiting the I/R-induced ROS production. This emetine-induced attenuation of oxidative stress could also be decreasing the SR Ca^{2+} leak. It has been reported that oxidation of ryanodine receptors (RyRs) enhances their activity promoting diastolic SR Ca^{2+} leak[30,31]. Moreover, oxidized RyRs have been detected in the reversible I/R injury[22]. Therefore, we cannot discard a contribution of a decreased ROS-driven SR Ca^{2+} leak in the emetine-mediated recovery of diastolic Ca^{2+} handling during I/R.

Emetine could also be restoring the normal ER-mitochondria Ca^{2+} transfer necessary to sustain oxidative phosphorylation. This Ca^{2+} transport takes place in contact sites between both organelles via a protein complex, which includes the inositol trisphosphate receptor (IP3R)[32,33]. The IP3R is an ER Ca^{2+} release channel stabilized by GRP78. Under ER stress conditions, the interaction between these two proteins is lost, leading to a reduce Ca^{2+} flux. Therefore, the cancellation of ER stress by emetine would ensure a proper ER-mitochondrial Ca^{2+} delivery by the GRP78-mediated facilitation of IP3R activity and it would simultaneously prevent a mitochondrial Ca^{2+} overload by the GRP78-mediated inhibition of the translocon, both effects resulting in the improvement of cellular bioenergetics.

4.1. Conclusions

Our results demonstrate for the first time that emetine prevents the ER stress and diastolic Ca^{2+} overload, enhancing contractility in an “ex-vivo” model of reversible I/R injury, even in the absence of a complete recovery of the oxidative state. The use of emetine in the present experiments

resulted successful in unmasking a crucial role of the ER Ca^{2+} efflux through the translocon in the stunned heart. Therefore, modulation of translocon permeability emerge as a yet not explored therapeutic approach to face dysfunctional consequences of the I/R injury. Knowing the cardiotoxicity of emetine administered at high doses or following chronic exposure[34,35], the development of new translocon blocking tools would be needed.

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FIGURE LEGENDS

Figure 1. Effects of emetine on expression of ER markers in stunned hearts. Total results of mRNA expression of: a) sXBP1 and b) GRP78 in non-ischemic hearts (Control) and hearts subjected to I/R in the absence (I/R) or presence of 1 μM of emetine (I/R + eme). c) Representative Western blots of phosphorylated and unphosphorylated forms of eIF2α (p-eIF2α and total-eIF2α, respectively) and GAPDH, and overall quantitative results expressed as p-eIF2α/total-eIF2α, in the same conditions. Data represent means ± SEM of 4-6 hearts/group. *P < 0.05 respect to Control. #P < 0.05 vs I/R.

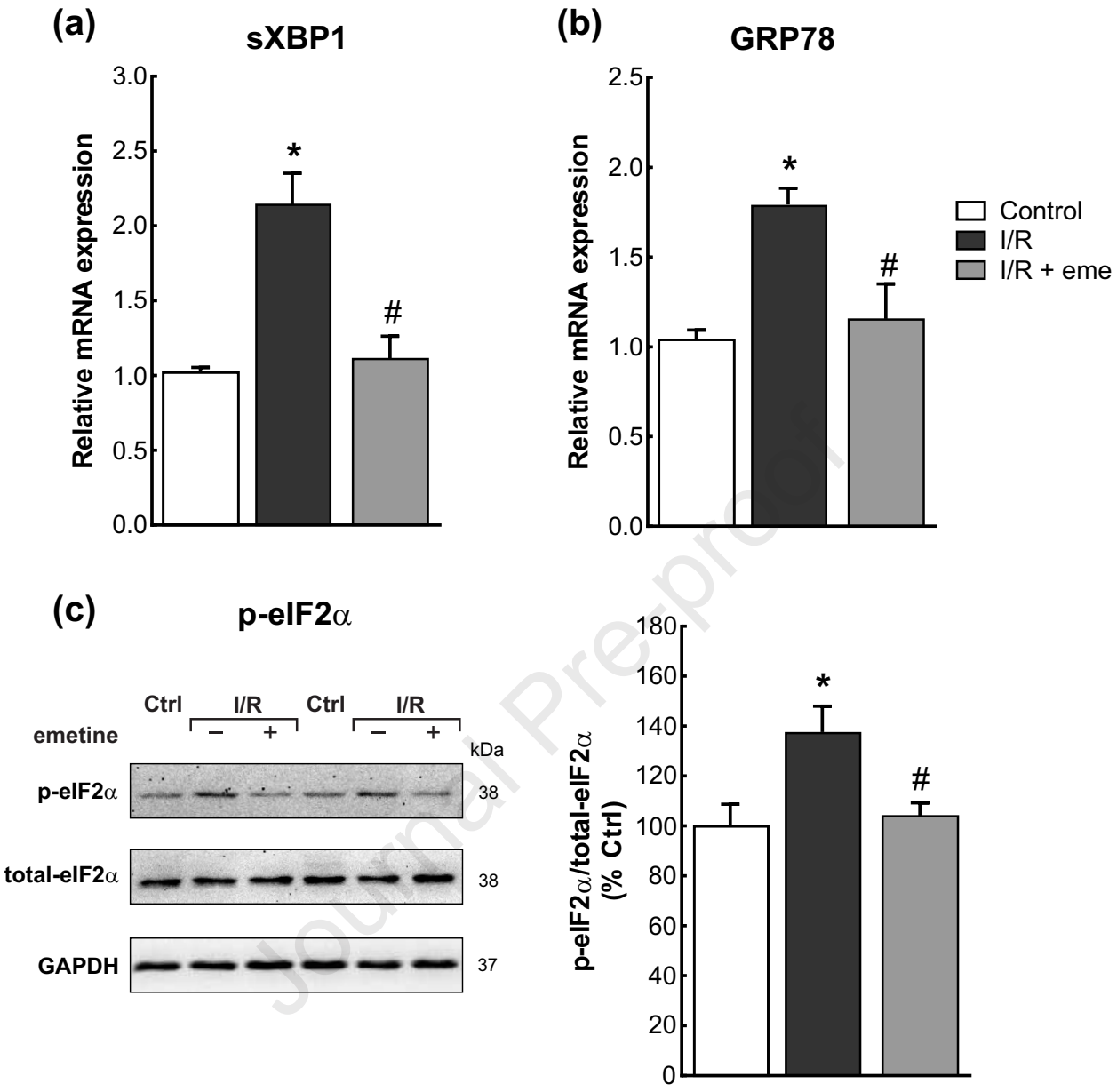
Figure 2. Effects of translocon inhibition on post-ischemic contractile recovery. a) Time course of left ventricular developed pressure (LVDP), b) maximal rate of pressure development (+dP/dt), c) left ventricular end diastolic pressure (LVEDP) and d) left ventricular relaxation time constant (Tau) during ischemia and reperfusion of non-treated hearts (I/R) and hearts perfused with 1 μM emetine (I/R + eme). LVDP, +dP/dt and LVEDP were expressed in absolute values and Tau was expressed as differences from pre-ischemic values. Data represent means ± SEM of 5-7 hearts/group. #P < 0.05 vs I/R.

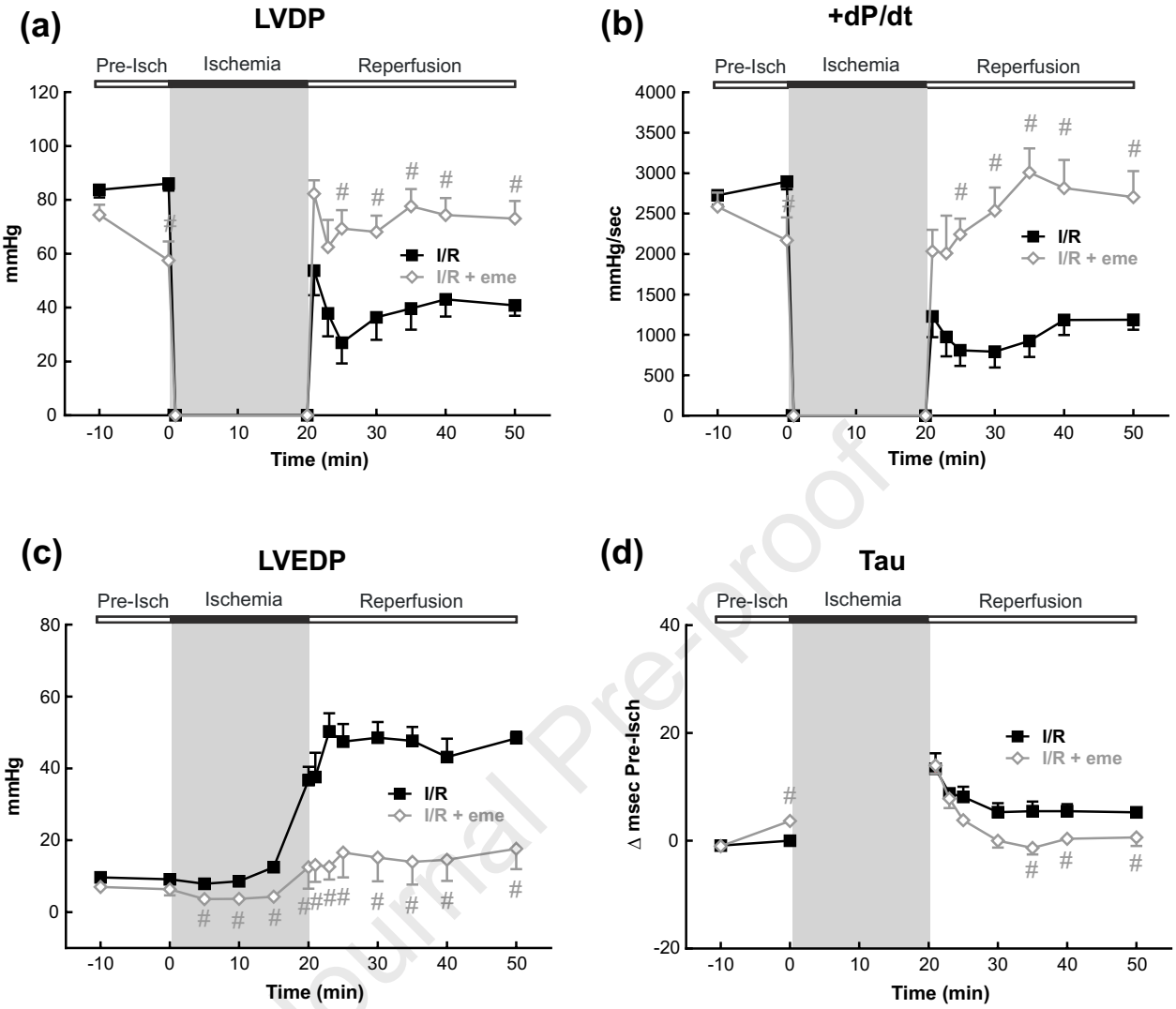
Figure 3. Comparison of the effects of emetine, TUDCA and 4-PBA on the post-ischemic mechanical recovery. a) Left ventricular developed pressure (LVDP), b) maximal rate of pressure development (+dP/dt), c) left ventricular end diastolic pressure (LVEDP) and d) relaxation time constant (Tau) at the end of the reperfusion period of hearts subjected to I/R in the absence (I/R) or presence of 1 μM emetine (I/R + eme), 30 μM TUDCA (I/R + TUDCA) and 3 mM 4-PBA (I/R + 4-PBA). LVDP, +dP/dt and LVEDP were expressed in absolute values and Tau was expressed as

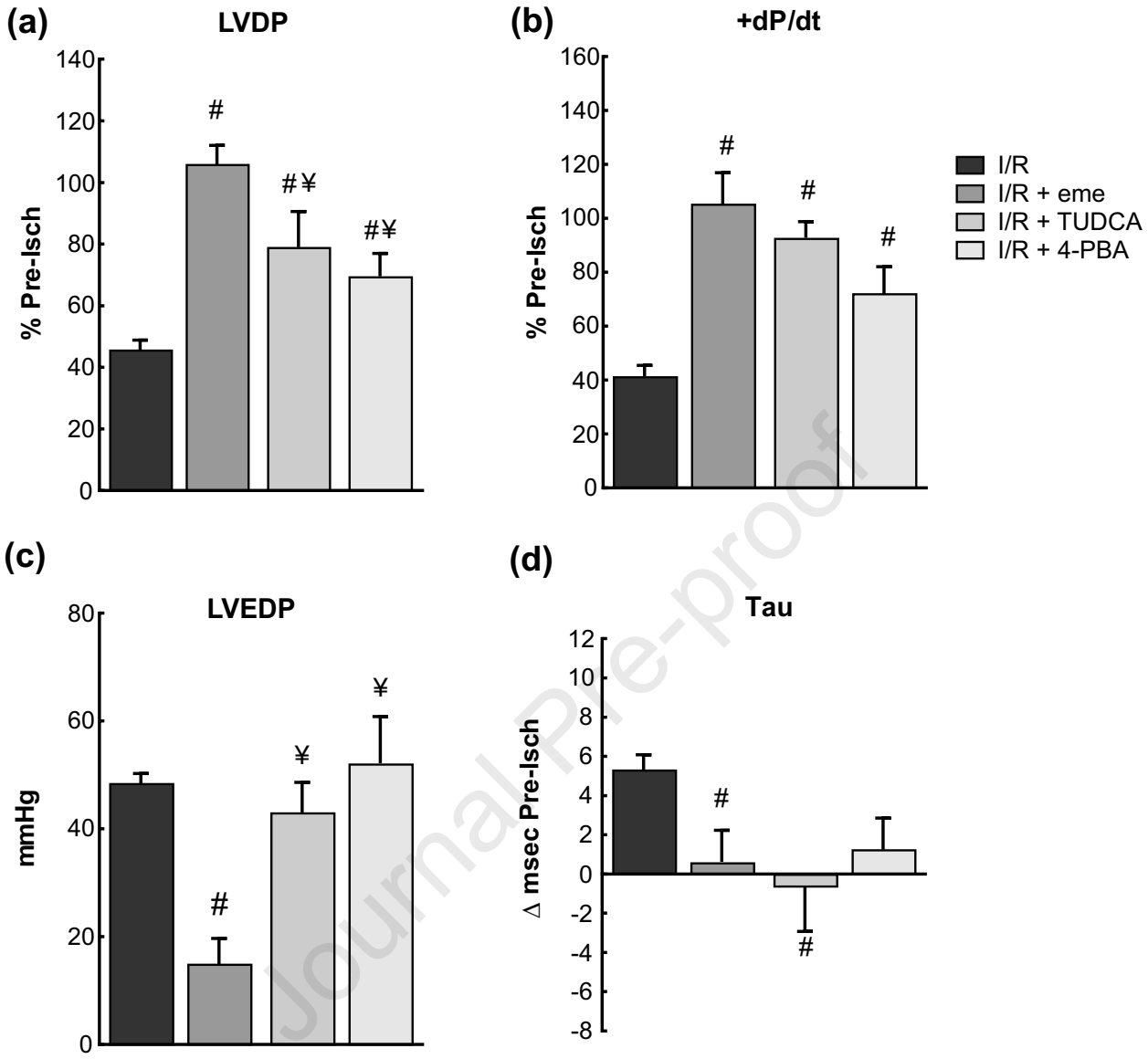
differences from pre-ischemic values. Data represent means ± SEM of 4-7 hearts/group. #P < 0.05 vs I/R. ¥ P<0.05 vs emetine.

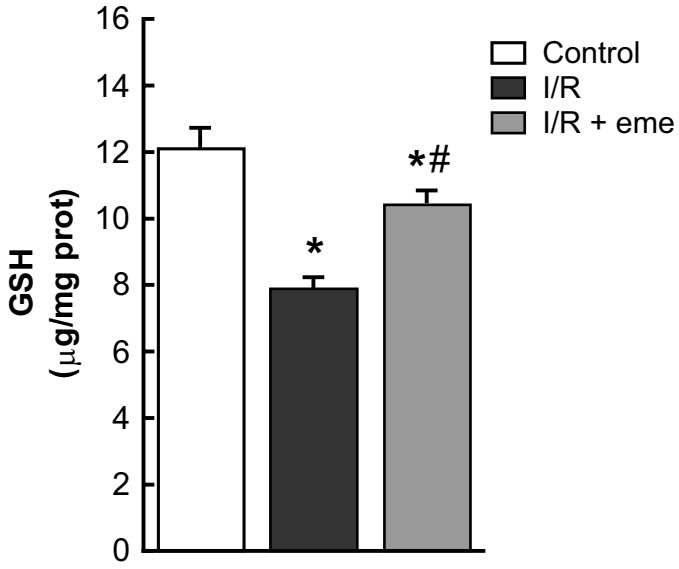
Figure 4. Effects of emetine on reduced glutathione (GSH) levels after I/R. Average levels of GSH levels (mean ± SEM of 5-6 hearts) measured in non-ischemic hearts (Control) and in I/R hearts in the absence (I/R) or presence of 1 µM of emetine (I/R + eme). *P < 0.05 respect to Control. #P < 0.05 vs I/R.

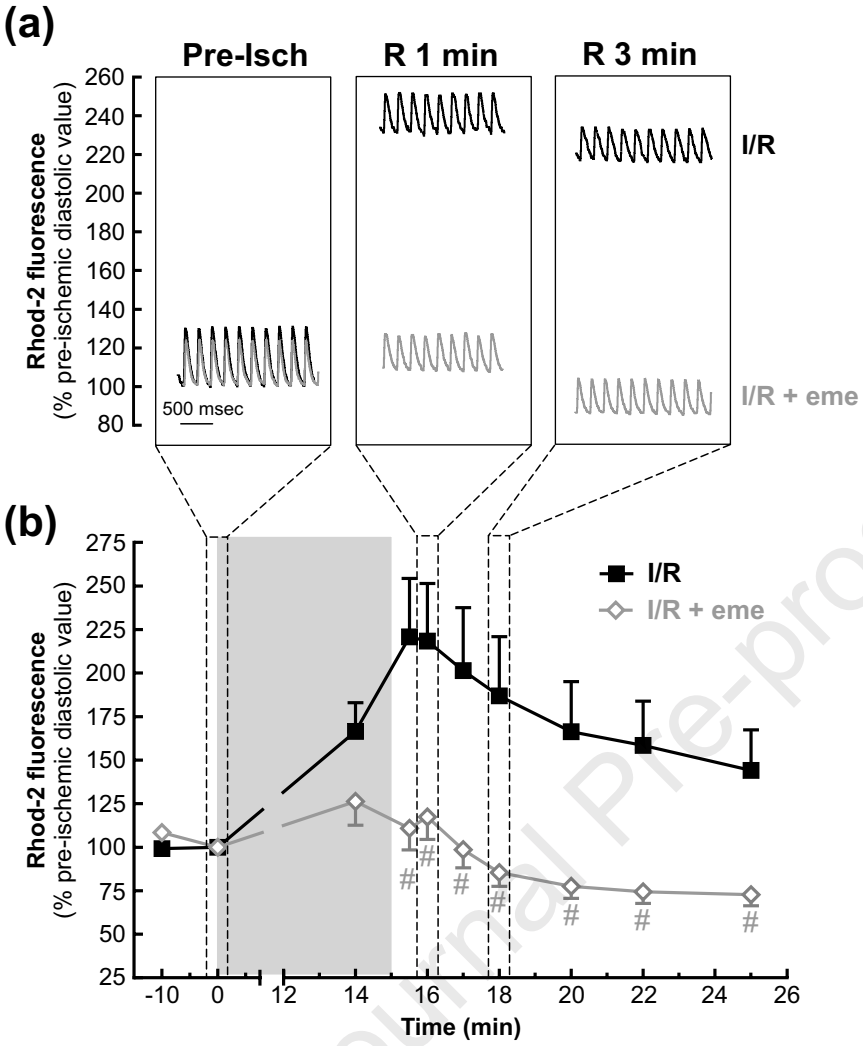
Figure 5. Effects of emetine on cytosolic Ca^{2+} during I/R. a) Typical recordings of intracellular Ca^{2+} transients measured in perfused mouse hearts loaded with Rhod-2 before ischemia (Pre-Isch) and at 1 and 3 min of reperfusion (R) in the absence (black traces) or the presence of 1 µM of emetine (red traces). b) Time course of diastolic Ca^{2+} of non-treated mouse hearts (I/R) and hearts perfused with 1 µM emetine (I/R + eme). Data represent means ± SEM of 5-7 hearts/group. #P < 0.05 vs I/R.











Highlights

- Blocking ER Ca^{2+} depletion via translocon suppressed the I/R-induced ER stress.
- ER Ca^{2+} leak contributes to Ca^{2+} mishandling and contractile impairment during I/R.
- Translocon inhibition emerges as a target to face deleterious consequences of I/R.

DECLARATION OF INTEREST

No conflicts of interest, financial or otherwise, are declared by the authors.