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Hyperosmotic stress promotes endoplasmic reticulum stress-dependent apoptosis in adult rat cardiac myocytes

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Abstract
In different pathological situations, cardiac cells undergo hyperosmotic stress and cell shrinkage. This change in cellular volume has been associated with contractile dysfunction and cell death. However, the intracellular mechanisms involved in hyperosmotic stress-induced cell death have not been investigated in depth in adult cardiac myocytes. Given that osmotic stress has been shown to promote endoplasmic reticulum stress (ERS), a recognized trigger for apoptosis, we examined whether hyperosmotic stress triggers ERS in adult cardiac myocytes and if so whether this mechanism mediates hyperosmotic stress-induced cell death. Adult rat cardiomyocytes cultured overnight in a hypertonic solution (HS) containing mannitol as the osmolite, showed increased expression of ERS markers, GRP78, CHOP and cleaved-Caspase-12, compared with myocytes in isotonic solution (IS), suggesting that hyperosmotic stress induces ERS. In addition, HS significantly reduced cell viability and increased TUNEL staining and the expression of active Caspase-3, indicative of apoptosis. These effects were prevented with the addition of the ERS inhibitor, 4-PBA, indicating that hyperosmotic stress-induced apoptosis is mediated by ERS. Hyperosmotic stress-induced apoptosis was also prevented when cells were cultured in the presence of a Ca^{2+}-chelating agent (EGTA) or the CaMKII inhibitor (KN93), suggesting that hyperosmotic stress-induced ERS is mediated by a Ca^{2+} and CaMKII-dependent mechanism. Similar results were observed when hyperosmotic stress was induced using glucose as the osmolite. We conclude that hyperosmotic stress promotes ERS by a CaMKII-dependent mechanism leading to apoptosis of adult cardiomyocytes. More importantly, we demonstrate that hyperosmotic stress-triggered ERS contributes to hyperglycemia-induced cell death.

Keywords Hyperosmotic stress · Endoplasmic reticulum stress · Apoptosis · CaMKII · Hyperglycemia

Introduction

The osmolarity of body fluids is tightly controlled under physiological conditions [1]. However, the volume of cells, including cardiomyocytes, can change under pathological conditions. During hyperglycemia, diabetes, heat stress and severe dehydration, cells suffer hyperosmotic stress and undergo shrinkage [2–4]. In the myocardium, this change in cell volume has been shown to result in contractile dysfunction and cell death [3, 5, 6]. However, the mechanisms underlying hyperosmotic stress-induced cell death of adult cardiac myocytes are far from being understood.

Cell shrinkage places a great amount of mechanical stress on the cytoskeleton [7] and recently it was recognized that mechanical stimuli can promote endoplasmic reticulum stress (ERS) in different tissues [8–12]. Interestingly, studies in non-cardiac cells indicate that osmotic stress also induces ERS [13, 14]. However, if hyperosmotic stress promotes ERS in adult cardiac myocytes has never been studied. ERS has been shown to contribute to the pathogenesis of several cardiovascular diseases such as cardiac hypertrophy, sepsis, hypertension, alcoholic cardiomyopathy and diabetes [15–20]. Moreover, ERS has been shown to contribute to pathological myocardial contractile dysfunction [21], and ERS inhibition has been shown to rescue cardiac contractile dysfunction [22] highlighting the causal role of ERS in cardiac pathology.

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The endoplasmic reticulum (ER) is a tubular organelle which is critically important for the proper folding of proteins. Various pathological conditions that disturb ER function can lead to the accumulation of unfolded or misfolded proteins in the ER lumen which induce ERS. To overcome ERS, the ER has a specific signaling pathway termed the unfolded protein response (UPR), which is an evolutionarily conserved pathway to accommodate protein-folding stress and restore cellular homeostasis [23]. The UPR leads to increased chaperone transcription such as the glucose-regulated protein 78 kDa (GRP78), which can contribute to improve the folding of the misfolded/unfolded proteins [24]. Moderate ERS can relieve cellular dysfunction and increase the possibility of survival, whereas excessive and prolonged ERS leads to apoptosis [25]. ERS-induced apoptosis is mediated by transcriptional induction of C/EBP homologous protein (CHOP/GADD153) and the subsequent activation of the caspase-12 dependent pathway [26–28]. Activation of ER-resident caspase-12 promotes the activation of cytoplasmic caspase-3 to trigger cellular apoptosis [29]. Taking into account that sustained or excessive ERS leads to apoptosis, we hypothesized that sustained hyperosmotic stress could promote apoptosis of adult cardiac myocytes by triggering ERS, contributing in this way, to cardiac pathological remodeling associated with disease. Since, it has been demonstrated that Ca^{2+}/calmodulin-activated protein kinase II (CaMKII) mediates ERS-induced cardiac apoptosis [30], we hypothesized that this kinase could be involved in cell death associated with hyperosmotic stress.

We show herein that hyperosmotic stress promotes ERS via a Ca^{2+} and CaMKII-dependent mechanism. Our results further show that ERS mediates hyperosmotic stress-induced apoptosis of adult rat cardiac myocytes. More importantly we show that hyperosmotic stress-triggered ERS may contribute to hyperglycemia-induced cell death.

Materials and methods

All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996) and approved by the Institutional Animal Care and Use Committee of La Plata University.

Reagent and antibodies

Urethane, collagenase, protease, M199 medium, taurine, L-NAME, MPG, EGTA, BSA, 4-PBA, KN-92 and KN-93 were obtained from Sigma-Aldrich (Sigma, St. Louis, MO, USA). Mannitol was purchased from Anedra (Tigre, Bs. As., Argentina) and glucose was obtained from Biopack (Bs.As., Argentina). HEPES was obtained from Affymetrix (Cleveland, Ohio, USA). SDS was purchased from Bio-Rad Laboratories (Osaka, Japan). Penicillin/Streptomycin was purchased from Gibco by Life Technologies (Grand Island, NY, USA) and laminin from ThermoFisher Scientific (Waltham, Massachussetts, USA).

Antibodies for CHOP and GRP78 were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). Antibodies for cleaved-caspase 12 and 3, and GAPDH were obtained from Life sciences Biotech (Millipore-Sigma, Burlington, Massachusetts, USA). Antibodies foro Bax and Bcl-2 were purchased from Abcam (Cambridge, UK).

Myocyte isolation

Male Wistar rats (200 to 300 g) were anaesthetised by an intra-peritoneal injection of urethane (1.2–1.4 g/kg) and hearts were excised when plane three of phase III of anaesthesia was reached.

Cardiac myocytes were isolated by collagenase-based enzymatic digestion using a previously described technique [31]. In brief, hearts were mounted on a Langendorff perfusion apparatus and were perfused retrogradely at a constant flow with a HEPES based salt solution (isolation solution—see below). When the coronary circulation had cleared of blood, perfusion was continued for 5 min with Ca^{2+}-free isolation solution containing 0.1 mM EGTA, and then for 15 min with isolation solution containing 0.05 mM $CaCl_2$, 0.5 mg/ml collagenase type II (300 U/ml), 0.025 mg/ml protease and 1.25 mg/ml BSA at 37 °C. After digestion was completed, the heart was disassembled and the ventricular tissue was mechanically dissociated with scissors. In this way a suspension of cells was obtained that were them subjected to 4 steps of decantation and re-suspension in isolation solutions with increasing concentration of $CaCl_2$, until a final concentration of 1 mM $CaCl_2$ was reached, cells were kept in suspension in this solution until use.

HEPES (isolation solution in mM): NaCl 146.2, KCl 4.7, $CaCl_2$ 1, HEPES 10, NaH_2PO_4. H_2O 0.35, $MgSO_4 \cdot 7H_2O$ 1.05, Glucose 10 (adjusted to pH 7.4 with NaOH).

Experimental protocol

Isolated cardiac myocytes were attached with laminin and cultured with sodium bicarbonate buffered Medium 199 supplemented with 5 mM of taurine and 1% of Penicillin/Streptomycin. This was used as isotonic solution (IS) and 131 mM of Mannitol was added to obtain a hypertonic solution (HS) with a difference in osmolarity of 131 mOsm respect to IS. Both solutions were supplemented with drugs and inhibitors according to the different experimental protocols used.

The osmolarity was chosen considering that similar values were used by us [32] and others [33–36]. Moreover, in

diabetic patients under severe decompensation, it has been reported that plasma osmolarity can reach values as high as the one used in the present study [37, 38].

When glucose was used as the osmolite, we used the solutions previously used by us [32] with minor modifications (see below).

Extracellular solution (mmol/l)	IS	ISGl	MHS	MHSGl
NaCl	91	91	91	91
KCl	5.3	5.3	5.3	5,3
$MgCl_2$	0.5	0.5	0.5	0.5
$CaCl_2$	1	1	1	1
HEPES	10	10	10	10
Mannitol	91.5	71.5	111.5	91.5
Glucose	10	30	10	30
pH	7.4	7.4	7.4	7.4
Osmolarity (mOsm)	309	309	329	329

Inmunodetection by Western blot analysis

Cardiac myocytes seeded into 25 mm Petri dishes were maintained during 60 min in IS or HS in the absence or presence of 3 mM of 4-phenylbutyric acid (4-PBA). Then, cells were collected and homogenized with lysis buffer. Proteins were measured by the Bradford method using BSA as standard. Homogenates (~100 µg of total protein per gel line) were seeded in a 10% SDS polyacrylamide gel and transferred to polyvinylidene difluoride membranes. Blots were probed overnight with different antibodies according to the different experiments (see below). Immunoreactivity was visualized by a peroxidase-based chemiluminescence detection kit (Amersham Biosciences) using a Chemidoc Imaging System. The signal intensity of the bands in the immunoblots was quantified by densitometry using Image J software (NIH).

Assessment of endoplasmic reticulum stress markers

Antibodies raised against CHOP (dilution 1:1000), GRP78 (dilution 1:500) and cleaved Caspase-12 (dilution 1:1000) were used as markers of ERS. Anti-GAPDH (dilution 1:10,000) was used for normalization.

Assessment of cell viability and apoptosis

After culturing cells overnight at 37 °C (16 to 20 h) in different isotonic solutions (IS, ISGl) or hypertonic solutions (HS, MHS, MHSGl) in absence or presence of different drugs, cells were evaluated morphologically, being classified as viable or non-viable according to their length-to-width ratio (≥ 3 were considered viable) [39]. From each culture, which was considered as an n equal to 1, at least 8 photographs per group were taken to count and classify the cells. Caspase-3 activity, used as an index of apoptosis, was determined using the Caspase-3 fluorescent substrate, Phiphilux (OncoImmunin; Gaithersburg, MD, USA). Briefly, 2.5 µmol/l Phiphilux was added to each culture dish at the end of the incubation period and allowed to incubate for 1 h at 37 °C. Myocytes were gently washed once and imaged under a fluorescence microscope [39]. The results were expressed as percentage of caspase-3 positive cells related to total number of cells. Apoptosis was also determined by TUNEL assay (In Situ Cell Death Detection Kit, TMR red, Roche, Mannheim, Germany). The TUNEL-positive cells were imaged under a fluorescence microscope (× 100 magnification) and counted in 10 random fields from each experimental situation. The results were expressed as percentage of TUNEL-positive cells related to total number of cells. DAPI (1 µg/ml, 4′,6-Diamidino-2-phenylindole dihydrochloride, Sigma, St. Louis, MO) was used for nuclear staining.

Antibodies raised against cleaved Caspase-3 (dilution 1:500), Bax (dilution 1:500) and Bcl-2 (dilution 1:500) were immunodetected by Western blot analysis. Anti-GAPDH (dilution 1:10,000) was used for normalization.

Assessment of intracellular Ca^{2+}

Isolated adult rat cardiac myocytes were loaded with 10 µM Fura-2 AM (ThermoFisher scientific) during 12 min. Rat myocytes were perfused for 15 min with a IS/HS solution and stimulated via 2-platinum electrodes on either side of the bath at 0.5 Hz. Fura-2 fluorescence was measured on an inverted microscope adapted for epi-fluorescence by an Ion Optix hardware. The ratio of the Fura-2 fluorescence (510 nm) obtained after exciting the dye at 340 and 380 nm was taken as an index of Ca^{2+} [31]. Time to 50% relaxation was used as an index of the rate of Ca^{2+} decay.

Statistical analysis

Unpaired Student *t* test, and One-way or Two-way ANOVA followed by the Tukey–Cramer or Student–Newman–Keuls post hoc tests were used for statistical comparisons when appropriate. Data was analysed using mean ± SEM. Differences were considered significant at $p \leq 0.05$.

Results

Hyperosmotic stress promotes endoplasmic reticulum stress in adult rat cardiac myocytes

The effect of maintaining adult cardiac myocytes in isotonic solution (IS) or hypertonic solution (HS) during 1 h on markers of endoplasmic reticulum stress (ERS) is depicted in Fig. 1.

Figure 1 illustrates representative blots and scatter plots showing that maintaining cardiac myocytes in HS significantly increases the expression of important protein components of the ERS response compared to cells maintained in IS: the chaperone glucose-regulated protein 78 (GRP78), C/EBP-homologous protein (CHOP) and cleaved Caspase-12 which are known to promote programmed cell death during the pro-apoptotic phase of the ERS response.

These results indicate that hyperosmotic stress promotes ERS and suggest a pro-apoptotic environment.

Hyperosmotic stress promotes adult cardiac myocyte apoptosis

It has been established that hyperosmotic stress promotes cell death in various cell types [40, 41], including neonatal cardiac myocytes [5, 6, 42]. To explore whether, hyperosmotic stress also promotes cell death in adult cardiac myocytes, we evaluated cell viability in adult cardiac myocytes exposed overnight to IS or HS. Figure 2 depicts representative images and scatter plots, showing that exposing adult rat cardiac myocytes to hyperosmotic stress decreases cell viability compared to cells maintained in IS.

To assess the mechanism responsible of cell death, we evaluated Caspase-3 activity as an index of apoptosis using a Caspase-3 fluorescent substrate and measuring cleaved Caspase-3 by the western blot technique. Apoptosis was also determined by TUNEL staining and by measuring the expression of the pro- and anti-apoptotic proteins Bax and Bcl-2, respectively (Bax/Bcl-2). Figure 2 depicts representative images, blots and scatter plots showing that hyperosmotic stress promotes an increase in Caspase-3 activity, in TUNEL-positive nuclei and in the Bax/Bcl-2 ratio indicating that hyperosmotic stress induces cell death at least in part by promoting apoptosis.

Endoplasmic reticulum stress mediates hyperosmotic stress-induced apoptosis

It has been reported that sustained ERS promotes cell death mainly by triggering apoptosis [28]. To evaluate the implication of ERS in hyperosmotic stress-induced cell death we performed experiments in the presence of 4-Phenylbutyric acid (4-PBA), a chemical chaperone known to inhibit ERS [43, 44].

Figure 3a shows that the increased expression of markers of ERS observed in presence of HS (Fig. 1) is prevented in the presence of 3 mM 4-PBA.

Figure 3b shows that the decrease in cell viability caused by hyperosmotic stress is cancelled in presence of 4-PBA. When apoptosis was analyzed by Caspase-3 activity, TUNEL staining and by the ratio of the pro- and anti-apoptotic proteins Bax and Bcl-2 respectively, there was no difference between cells maintained in IS + 4-PBA and HS + 4-PBA suggesting that ERS mediates apoptotic cell death under hyperosmotic conditions.

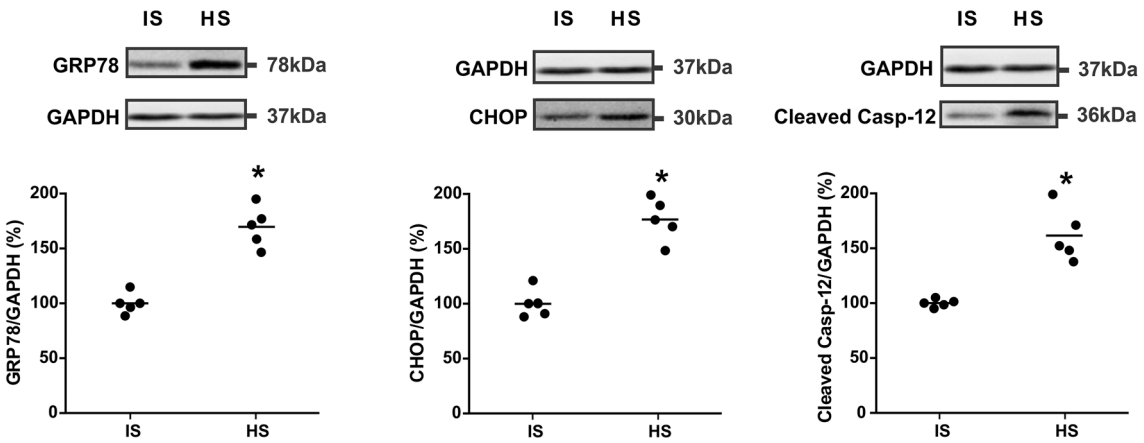

Fig. 1 Hyperosmotic stress promotes endoplasmic reticulum stress in adult rat cardiac myocytes. Representative blots and scatter plots showing that in adult cardiac myocytes, HS significantly increases the protein expression of GRP78, CHOP and cleaved Caspase-12 compared to IS. Scatter plot shows values from five independent myocyte isolations (five hearts), where horizontal bars represent means; *$p \leq 0.05$ versus cardiac myocytes maintained in IS

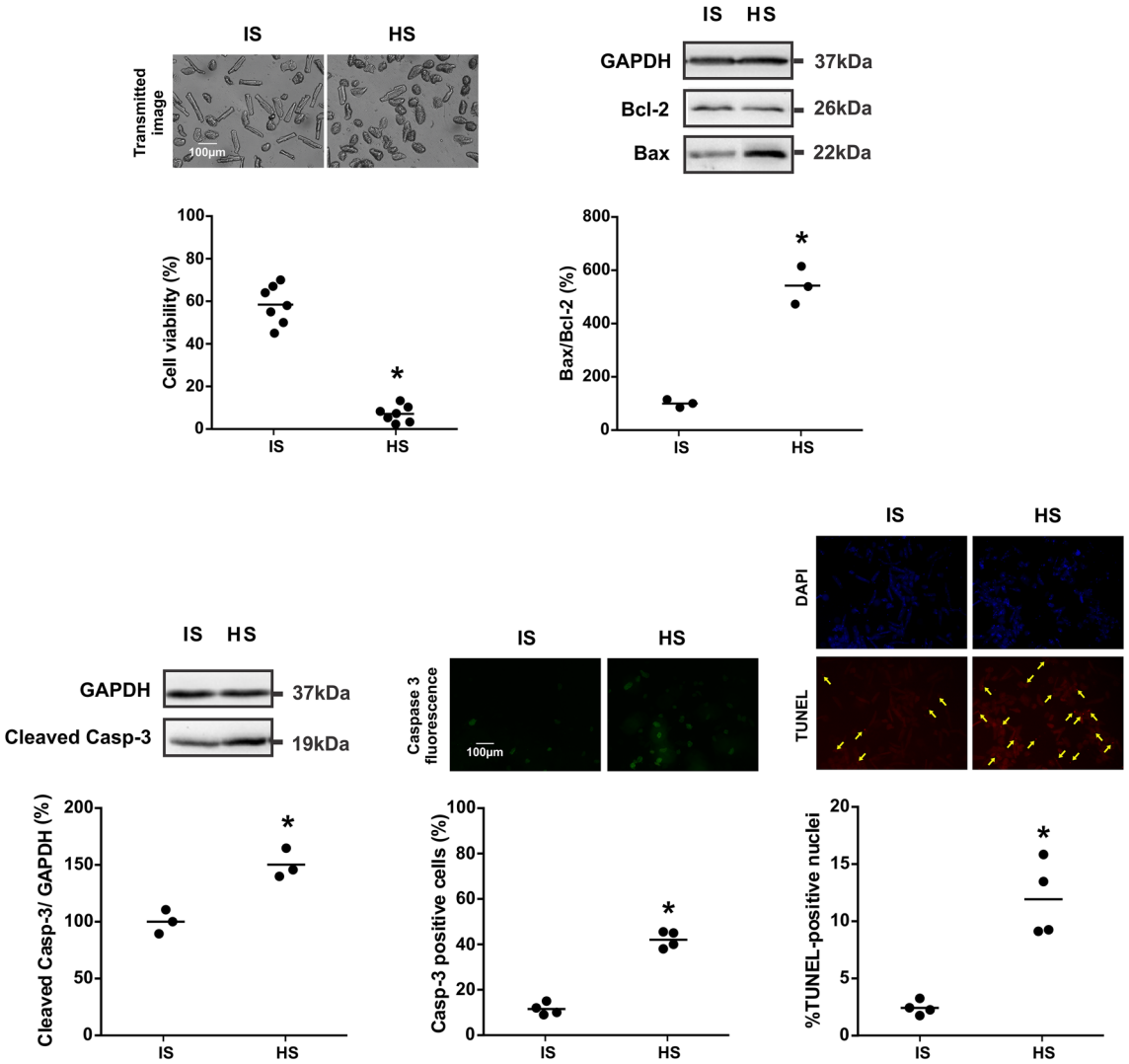

Fig. 2 Hyperosmotic stress promotes adult cardiac myocyte apoptosis. Representative images, blots and scatter plots, showing that exposing adult cardiac myocytes to HS decreases cell viability compared to cells maintained in IS. In addition, HS promotes an increase in Caspase-3 activity measured by a Caspase-3 fluorescent substrate and in cleaved Caspase-3 measured by the western blot technique. Western blot analysis also shows an increase in the ratio between pro- and anti-apoptotic proteins Bax and Bcl-2, respectively (Bax/Bcl-2). Typical example and overall results of the increase in TUNEL-positive cells produced by hyperosmotic stress in rat myocytes. Scatter plot shows values from 3 to 7 independent myocyte isolations (3–7 hearts), where horizontal bars represent means; *$p \leq 0.05$ versus cardiac myocytes maintained in IS

Ca^{2+} signaling mediates hyperosmotic stress-induced cell death

Recent evidence indicates that hyperosmotic stress is associated with an increase in the intracellular Ca^{2+} transient [3], with the generation of reactive oxygen species (ROS) [45] and with an increase in nitric oxide (NO) production [32, 46]. Moreover, it is known that Ca^{2+} overload, ROS and NO can promote ERS and consequently cell death [47, 48]. Based on this, to explore the mechanisms underlying hyperosmotic stress-induced ERS and cell death, we performed experiments in which we assessed cell viability of adult cardiac myocytes exposed to IS/HS in the presence of either, the NO synthase inhibitor (L-NAME, 2.5 mM), the scavenger of ROS (*N*-2-mercaptopropionylglycine (MPG, 2 mM)), or the Ca^{2+}-chelating agent (EGTA, 10 mM).

Figure 4a depicts representative images and scatter plots showing that overnight cell culture of cardiac myocytes exposed to HS in presence of L-NAME or MPG suffer the same decrease in cell viability as the one observed with HS in the absence of drugs that was shown in Fig. 2, suggesting that ROS and NO are not mechanistically involved in cell death promoted by hyperosmotic stress-induced ERS.

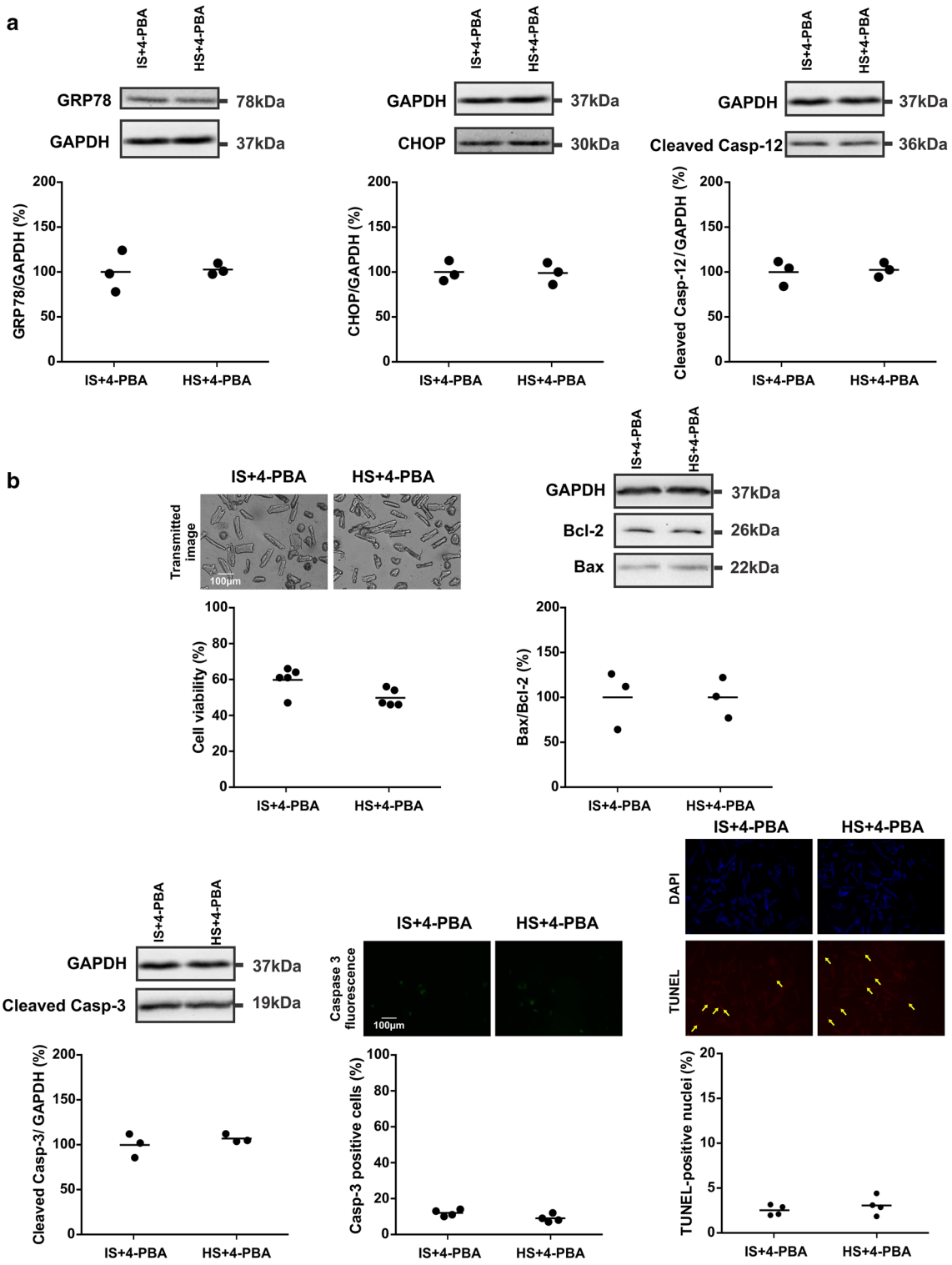

Fig. 3 Endoplasmic reticulum stress mediates hyperosmotic stress-induced apoptosis. **a** Representative blots and scatter plots showing that in presence of 4-PBA, HS does not increase the protein expression of GRP78, CHOP and cleaved Caspase-12 compared to IS + 4-PBA. Scatter plot shows values from three independent myocytes isolation (three hearts), where horizontal bars represent means. **b** Representative images, blots and scatter plots, showing that in presence of 4-PBA, HS does not reduce cell viability, or increase Caspase-3 activity or the Bax/Bcl-2 ratio or the percentage of TUNEL-positive nuclei. Scatter plot shows values from 3 to 5 independent myocyte isolations (3–5 hearts), where horizontal bars represent means

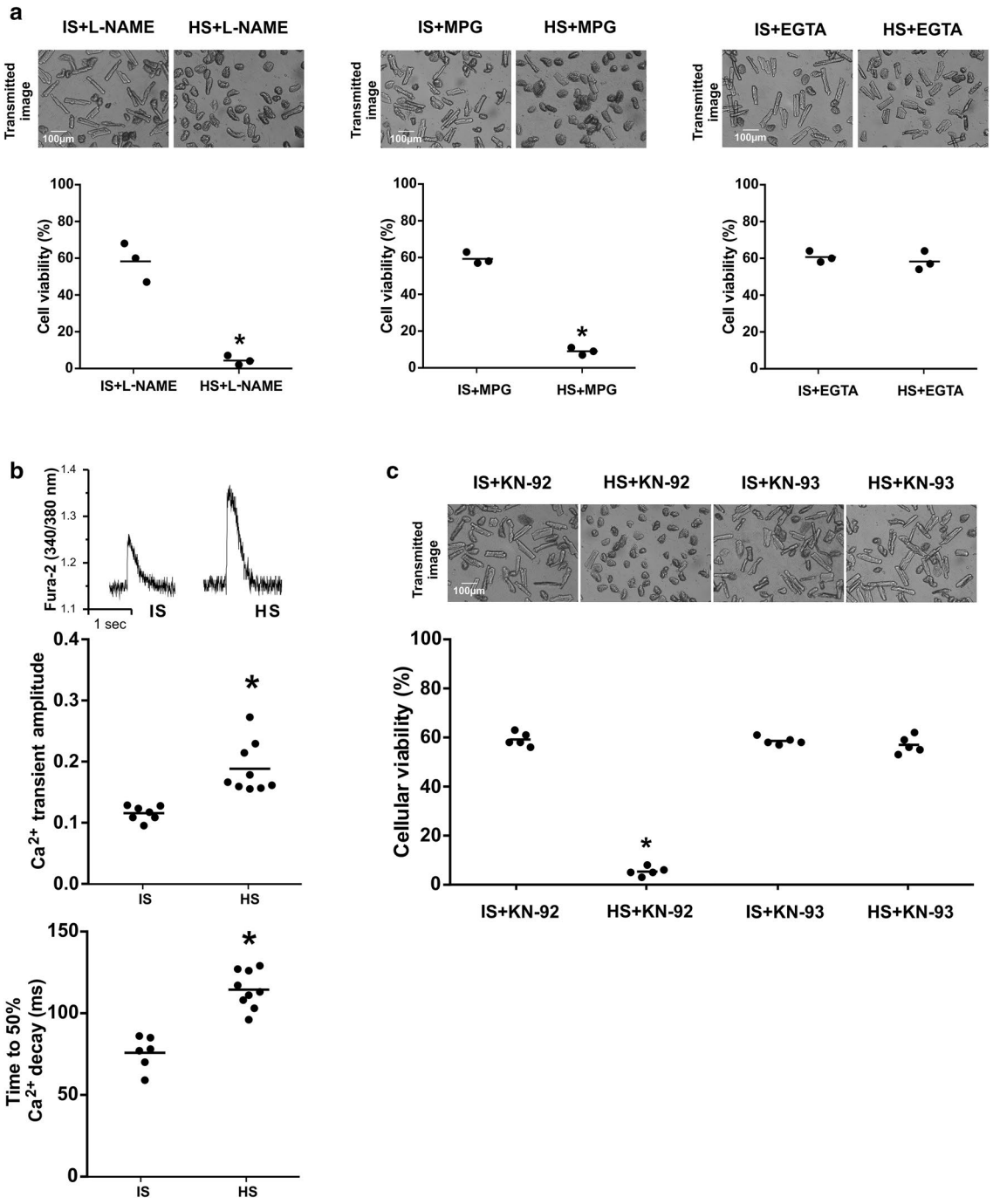

Fig. 4 Ca^{2+} signaling mediates hyperosmotic stress-induced cell death. Representative images and scatter plots showing the effect of exposing cardiac myocytes to IS or HS in absence or presence of L-NAME, MPG or EGTA on cell viability. Scatter plot shows values from three independent myocyte isolations (three hearts), where horizontal bars represent means. *$p \leq 0.05$ versus cardiac myocytes maintained in IS + L-NAME/IS + MPG. b: Typical tracings showing the effect of hyperosmotic stress on the amplitude and time to 50% decay of the Ca^{2+} transient of cardiomyocytes superfused with IS and after 15 min of superfusion with HS. Scatter plot shows values from three independent myocyte isolations (three hearts), where horizontal bars represent means; *$p \leq 0.05$ versus cardiac myocytes maintained in IS. **c** Representative images and scatter plot showing the effect of IS or HS in presence of the CaMKII inhibitor (KN-93) or the inactive KN-93 analog (KN-92) on cardiac myocyte viability. CaMKII inhibition completely prevented hyperosmotic stress-induced cell death whereas KN-92 failed to protect cells from HS-induced apoptosis. Scatter plot shows values from five independent myocyte isolations (five hearts), where horizontal bars represent means. *$p \leq 0.05$ versus cardiac myocytes maintained in IS + KN-92/IS + KN-93, HS + KN-93

When the same experiments were performed incubating cells with HS in the presence of a Ca^{2+} chelator (EGTA), no differences in cell viability could be observed compared to cells exposed to IS plus EGTA suggesting that an increase in Ca^{2+} could be the trigger for ERS associated with hyperosmotic stress. Indeed, consistent with results of others [3], Fig. 4b shows that superfusing cells with HS produces a significant increase in the amplitude and duration of the intracellular Ca^{2+} transient supporting the contention that hyperosmotic stress-induced ERS is mediated by a Ca^{2+} dependent mechanism.

Ca^{2+}/calmodulin-activated protein kinase II (CaMKII) is a serine/threonine protein kinase activated in response to an increase in intracellular Ca^{2+} [49] and is a common intermediary of diverse apoptosis-inducing insults [39, 50, 51]. Moreover, it has been demonstrated that this kinase mediates ERS-induced cardiac apoptosis [30]. Based on this background, we examined if this kinase was involved in cell death observed under our experimental conditions.

Figure 4c depicts representative images and scatter plots showing that exposing cardiac myocytes to HS in presence of 2.5 µM of the CaMKII inhibitor, KN-93, completely prevents hyperosmotic stress-induced cell death whereas 2.5 µM of KN-92 (the inactive KN-93 analog) fails to protect cells from hyperosmotic stress-induced injury.

Taken together, the above results show that hyperosmotic stress evoked by a difference in osmolarity of 131 mOsm can induce CaMKII-dependent ERS which in turn promotes apoptotic cell death.

Even though it has been described that certain pathologies can reach an increase in osmolarity similar to the one used in this study [37, 38], milder changes in osmolarity have also been documented [38, 52–54]. Thus, we examined whether a smaller increase in osmolarity (+20 mM), was able to promote ERS and induce cell death.

Mild hyperosmotic stress promotes endoplasmic reticulum stress-induced cell death

Figure 5 depicts representative images and scatter plots of cell viability of adult cardiac myocytes maintained overnight in IS or in a mild hypertonic solution (MHS) in the absence or presence of 4-PBA, KN-92 or KN-93. Increasing osmolarity by 20 mOsm with mannitol (MHS) produces the same degree of decrease in cell viability that was observed with a difference of 131 mOsm (Fig. 2). Moreover, cell death was also reversed by the ERS inhibitor, 4-PBA, and by the CaMKII inhibitor, KN-93, suggesting that a smaller degree of hyperosmolarity is also capable of promoting CaMKII-dependent ERS-induced cell death.

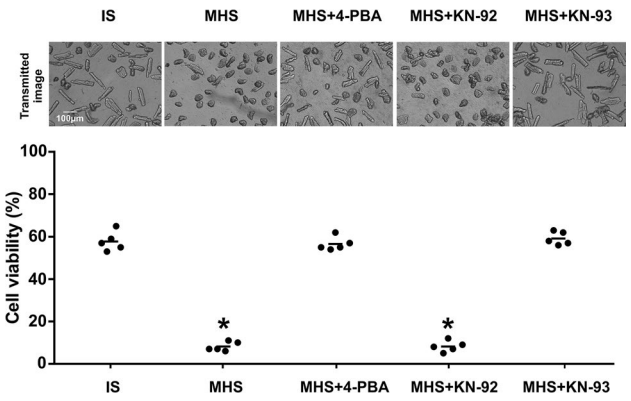

Fig. 5 Mild hyperosmotic stress promotes endoplasmic reticulum stress-induced cell death. Representative images and scatter plot of cell viability of cardiac myocytes maintained overnight in IS or MHS in absence or presence of 4-PBA, KN-92 and KN-93. Scatter plot shows values from five independent myocyte isolations (five hearts), where horizontal bars represent means. *$p \leq 0.05$ versus cardiac myocytes maintained in IS, MHS + 4-PBA, MHS + KN-93

Glucose-induced hyperosmolarity promotes cell death via CaMKII-dependent endoplasmic reticulum stress

It is known that patients with diabetes mellitus have an increase in plasma osmolarity due to an increase in glucose. To evaluate the effect of high glucose induced hyperosmolarity on the viability of adult cardiomyocytes, we performed experiments in which the hyperosmolar solution had the same osmolarity as MHS used in the experiments shown in Fig. 5, but using glucose instead of mannitol as the osmolite (MHSGl).

Figure 6 depicts representative images and scatter plots of cell viability of cardiac myocytes maintained overnight in MHSGl or ISGl (IS with the same amount of glucose as MHSGl to serve as control) in the absence or presence of 4-PBA, KN-92 or KN-93 showing that an increase in osmolarity due to an increase in glucose (MHSGl) produces the same degree of cell death as that induced by an increase in mannitol (MHS). This effect was canceled in the presence of 4-PBA or KN-93 suggesting that when glucose is the osmolite, cell death is also mediated by CaMKII-dependent ERS.

Discussion

In different pathological situations, such as hyperglycemia, ischemia, septic shock, diabetic coma, and severe dehydration, cardiomyocytes may undergo osmotic stress [2–4, 55]. In particular, an increase in extracellular osmolarity has many damaging effects on cells by promoting water flux out of the cell, cell shrinkage, intracellular dehydration, mechanical stress and membrane deformation [4, 56].

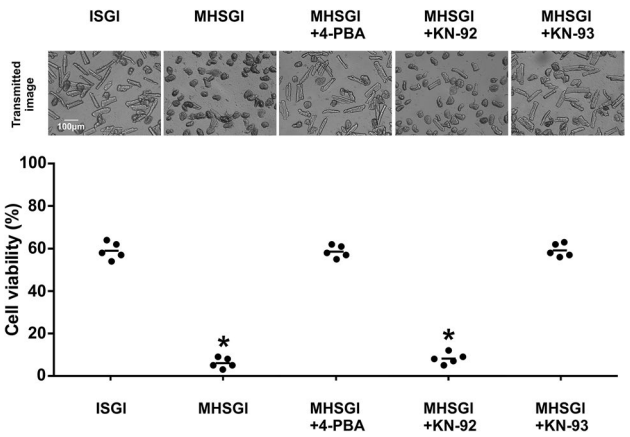

Fig. 6 Glucose-induced hyperosmolarity promotes cell death via CaMKII-dependent endoplasmic reticulum stress. Representative images and scatter plot of cell viability of adult cardiac myocytes maintained overnight in ISGl or MHSGl in the absence or presence of 4-PBA, KN-92 or KN-93. Scatter plot shows values from five independent myocyte isolations (five hearts), where horizontal bars represent means. *$p \leq 0.05$ versus cardiac myocytes maintained in ISGl, MHSGl + 4-PBA, MHSGl + KN-93

Indeed, in the myocardium, hyperosmotic stress has been associated with ionic remodeling, contractile dysfunction and neonatal cardiac myocyte cell death [3, 5, 6, 32]. However, the underlying mechanisms involved in these deleterious actions of hyperosmotic stress are far from being understood. Interestingly, mechanical stress has been shown to promote ERS and apoptosis of cardiac myocytes [57] and studies in non-cardiac cells have shown that hyperosmotic stress can modulate ER function, triggering the UPR. Thus, we hypothesized that hyperosmotic stress-could trigger the UPR in adult cardiac myocytes and mediate apoptosis. The present study shows, for the first time, that hyperosmotic stress promotes CaMKII-dependent ERS in isolated adult rat ventricular cardiomyocytes. Moreover, we show that sustained hyperosmotic stress-induced ERS promotes adult cardiomyocyte apoptosis which could contribute to cardiac contractile dysfunction associated with pathological situations that are accompanied with osmotic stress. Consistently, our results show that hyperosmotic stress-triggered ERS mediates hyperglycemia-induced cell death, possibly contributing to the evolution of the diabetic heart to diabetic cardiomyopathy.

Hyperosmotic stress promotes ERS in adult rat cardiac myocytes

The ER comprises a complex membranous network found in all eukaryotic cells. It plays a crucial role in the folding of secretory and membrane proteins, calcium homeostasis, and lipid biosynthesis [58–60]. ERS can occur in response to a wide variety of cellular stressors that disrupt ER function leading to the accumulation of unfolded and misfolded proteins in the ER. Initially, ER transmembrane sensors detect the accumulation of unfolded proteins and activate transcriptional and translational pathways that deal with unfolded and misfolded proteins, known as the UPR. ERS is initially protective, aiming to restore ER homeostasis. However, prolonged periods of ERS can be deleterious and damaging, causing cells to undergo apoptotic cell death [61, 62]. Recently, adaptive and proapoptotic pathways of the UPR have been implicated in the pathophysiology of cardiovascular diseases, including ischemia and reperfusion, diabetes and sepsis [63–65], all of which are also associated with osmotic misbalance [3, 66, 67]. Interestingly, mechanical stimuli have been recognized as an extracellular stress causing ERS in different tissue cells [8–12] and recent studies in non-cardiac cells indicate that osmotic stress can also induce ERS [13, 68]. However, if hyperosmotic stress promotes ERS, has not been studied in adult cardiac myocytes. We show herein that in the presence of HS, markers of ERS (GRP78, CHOP and cleaved-Caspase 12 are significantly enhanced in adult rat cardiac myocytes (Fig. 1), providing the first evidence showing that hyperosmotic stress promotes ERS in the adult heart. Several lines of evidence demonstrate that hyperosmotic stress promotes cell death in different cell types [40, 41] and in the heart, hyperosmotic stress has been shown to promote cell death in neonatal cardiac myocytes [5, 6]. Since it is recognized that sustained ERS leads to cell death by apoptosis, we evaluated whether ERS was involved in hyperosmotic stress-induced cardiomyocyte death. We first evaluated the impact of hyperosmotic stress on adult cardiac myocyte viability. Figure 2 shows that culturing adult cardiac myocytes in hypertonic solution (HS) decreases cell viability compared to cells maintained in isotonic solution (IS). Figure 2 also shows that the decrease in viability induced by HS is associated with an increase in TUNEL positive cells indicative of apoptosis. Consistently, HS increased caspase-3 activity, one of the end effectors of the apoptotic cascade, and increased Bax/Bcl-2 ratio which would suggest the involvement of mitochondria in hyperosmotic stress-induced apoptotic cardiomyocyte death. Taken together, these results suggest that hyperosmotic stress promotes cell death, at least in part, by triggering apoptosis of adult rat cardiac myocytes. Next, we evaluated if ERS was involved in the reduction of viability of cardiac myocytes exposed to hyperosmotic stress. As shown in Fig. 3a, treatment with a chemical chaperone that inhibits ERS (4-PBA) not only prevented the increase in the expression of ERS markers induced by HS but also prevented the decrease in viability and the increase in apoptotic indexes associated with hyperosmotic stress (Fig. 3b). Indeed, the ability of 4-PBA to prevent hyperosmotic stress-induced apoptosis supports the contention that ERS is triggered by hyperosmotic stress and that this altered ER function is causally

involved in adult cardiac myocyte apoptosis induced under hyperosmotic conditions.

The mechanisms that trigger ERS are still not completely understood. Previous reports have demonstrated that an unbalanced intracellular Ca^{2+} homeostasis caused by mechanical stimulation could lead to ERS [8, 12, 69]. Another potential mediator is NO, whose production has been shown to be increased in mechanically overloaded cells, contributing to ERS activation [48]. In addition, it has been demonstrated that ROS production could produce ERS [47]. Consistently, it has been shown that hyperosmotic stress can also increase intracellular Ca^{2+} [70], NO release [32] and ROS production [45]. Thus, we examined whether Ca^{2+}, NO or ROS were the signaling molecules involved in triggering ERS under hyperosmotic stress in adult rat cardiac myocytes. Using hyperosmotic stress-induced cell death as a readout of hyperosmotic stress-induced ERS we show that both the NO synthase inhibitor, L-NAME, or the ROS scavenger, MPG, fail to prevent hyperosmotic stress-induced cell death suggesting that neither NO nor ROS are mechanistically involved in triggering ERS during hyperosmotic stress. In contrast, when adult cardiac myocytes were incubated with HS in presence of the Ca^{2+} chelator, EGTA, cells were protected from hyperosmotic stress-induced cell death (Fig. 4a), suggesting that altered Ca^{2+} handling could be the trigger for ERS associated with hyperosmotic stress. In support of this contention, HS induced a significant increase in cardiomyocyte intracellular Ca^{2+} transient (Fig. 4b). These results are consistent with those of a previous study that concluded, using EGTA to reduce intracellular Ca^{2+}, that hyperosmotic stress-induced apoptosis of neonatal cardiomyocytes was a Ca^{2+}-dependent process [71]. Nevertheless, how this Ca^{2+} signal triggers ERS in adult cardiac myocytes remains elusive. Notably, in a recent study Ren et al. showed, using a mouse model of ERS induced by tunicamycin, that activation of Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII) mediates ERS-induced apoptosis [30]. CaMKII is a ubiquitous threonine/serine kinase that is canonically activated by the elevation of intracellular Ca^{2+}. However, more recently ROS and NO have also been shown to activate the kinase [72–74]. Interestingly, CaMKII has been shown to be a common intermediate through which diverse death-inducing stimuli trigger cardiomyocyte apoptosis [39, 51, 75]. Thus, we hypothesized that the hyperosmotic stress-induced increase in Ca^{2+} could activate CaMKII which would mediate ERS. Using the CaMKII inhibitor, KN-93 and its inactive analog KN-92 we were able to suppress or not affect, the reduction in cell viability induced by hyperosmotic stress, respectively (Fig. 4c) providing robust evidence supporting that CaMKII mediates ERS-induced cardiomyocyte apoptosis under hyperosmotic conditions in adult cardiac myocytes. Our results showing that neither MPG nor L-NAME inhibit hyperosmotic stress-induced cell death further suggest that it is the increase in Ca^{2+} and not ROS or NO that mediates CaMKII activation under our experimental conditions.

The present results, showing that an increase in osmolarity of 131 mOsm promotes ERS-induced adult cardiomyocyte apoptosis are relevant given that during certain pathologies osmolarity can reach similar levels [37, 38]. However, it has been described that under some physiopathological situations, milder changes in osmolarity can also occur [38, 52–54]. Thus, we examined whether a smaller increase in osmolarity (20 mOsm above IS), was able to promote ERS and induce cell death. Interestingly, the results shown in Fig. 5 indicate that even a mild increase in osmolarity (MHS) is sufficient to trigger CaMKII/ERS-dependent cardiomyocyte apoptosis.

Physiopathological relevance of hyperosmotic stress-induced cardiomyocyte death

Diabetes evolves to diabetic cardiomyopathy which is a major cardiovascular complication that causes mortality and morbility in patients [76, 77]. Apoptosis has been shown to contribute to diabetic cardiomyopathy [78, 79]. However, the mechanisms underlying cardiomyocyte apoptosis of the diabetic myocardium are not completely understood. Diabetes is a complicated and progressive disease characterized, among other things, by hyperglycemia, increased ROS production and hyperosmotic stress [80, 81]. In addition, it has been shown that ERS is involved in the development of diabetic cardiomyopathy, at least in part, by triggering apoptosis [82, 83] and CaMKII has been implicated in promoting cardiomyocyte apoptosis in several models of diabetes [84, 85]. Taken together, these findings support the contention that in diabetes, hyperglycemia-induced hyperosmotic stress could contribute to the pathogenesis of the disease by promoting CaMKII-dependent ERS that would trigger apoptosis. To test this hypothesis we performed experiments in an in vitro model of hyperglycemia. We found that hyperosmotic stress produced by elevated glucose (+20 mM, Fig. 6—MSHGl) significantly reduced cardiomyocyte viability and that this reduction was abrogated by the ERS inhibitor, 4-PBA, and by the CaMKII inhibitor, KN-93, but not by its inactive analog KN-92. These results suggest that osmotic stress produced by hyperglycemia promotes CaMKII-dependent ERS leading to cardiac myocyte cell death. It is possible that high glucose could induce ERS and apoptosis by mechanisms independent of its osmotic behavior. However, our experiments performed with elevated glucose but maintained at isosmolar conditions by reducing mannitol (ISGl; see methods for solution composition), showed similar levels of cell viability compared to those in IS prepared with mannitol alone (Fig. 5) implying that glucose-induced osmotic stress in itself is capable of promoting cell death probably by triggering ERS-dependent apoptosis. Interestingly, previous studies have shown that hyperosmotic stress induced by mannitol does not

mimic elevated glucose-induced prolongation of relaxation, suggesting that hyperosmotic stress does not contribute to glucose-induced contractile dysfunction [86, 87]. The reason for this discrepancy is no apparent to us however, it is possible that a small degree of hyperosmotic stress induced by mannitol is not sufficient to impact on single myocyte contractile function, but if sustained in time hyperosmotic stress could contribute to myocardial contractile dysfunction by triggering an ERS-dependent apoptotic response.

Hyperglycemia-induced ROS production has also been shown to promote ERS-dependent apoptosis [88]. However, our results depicted in Fig. 4a showing that the ROS scavenger did not prevent HS-induced cell death suggest that hyperosmotic stress can contribute to hyperglycemia induced cell death by a mechanisms that requires ERS but is independent of enhanced ROS production. Taken together, these results reveal that hyperosmotic stress produced by hyperglycemia can promote CaMKII-dependent ERS leading to cardiac myocyte cell death, highlighting that hyperosmotic stress could be an important contributor to cardiac adverse remodeling associated with diabetic cardiomyopathy.

In summary, our results show, for the first time, that hyperosmotic stress promotes ERS by a CaMKII-dependent mechanism leading to apoptosis of adult cardiomyocytes. More importantly, we demonstrate that CaMKII-dependent ERS induced by hyperosmotic stress mediates hyperglycemia-induced cell death. Thus, cardiac specific CaMKII inhibition could result a promising therapeutic strategy to alleviate cardiac myocyte loss in patients that develop diabetic cardiomyopathy. Future studies delineated to understand how CaMKII triggers ERS under hyperosmotic stress conditions could provide novel pharmacological targets for the treatment of cardiac dysfunction associated with pathologies that are coupled with hyperosmotic stress.

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Compliance with ethical standards

Conflict of interest The author declares that they have no conflict of interest.

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