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ORIGINAL CONTRIBUTION

AMPK-dependent nitric oxide release provides contractile support during hyperosmotic stress

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Abstract

In different pathological situations, cardiac cells undergo hyperosmotic stress (HS) and cell shrinkage. This change in cellular volume has been associated with contractile dysfunction and cell death. Given that nitric oxide (NO) is a well-recognized modulator of cardiac contractility and cell survival, we evaluated whether HS increases NO production and its impact on the negative inotropic effect observed during this type of stress. Superfusing cardiac myocytes with a hypertonic solution (HS: 440 mOsm) decreased cell volume and increased NO-sensitive DAF-FM fluorescence compared with myocytes superfused with an isotonic solution (IS: 309 mOsm). When cells were exposed to HS in addition to different inhibitors: L-NAME (NO synthase inhibitor), nitroguanidine (nNOS inhibitor), and Wortmannin (eNOS inhibitor) cell shrinkage occurred in the absence of NO release, suggesting that HS activates nNOS and eNOS. Consistently, western blot analysis demonstrated that maintaining cardiac myocytes in HS promotes phosphorylation and thus, activation of nNOS and eNOS compared to myocytes maintained in IS. HS-induced nNOS and eNOS activation and NO production were also prevented by AMPK inhibition with Dorsomorphin (DORSO). In addition, the HS-induced negative inotropic effect was exacerbated in the presence of either L-NAME, DORSO, ODQ (guanylate cyclase inhibitor), or KT5823 (PKG inhibitor), suggesting that NO provides contractile support via a cGMP/PKG-dependent mechanism. Our findings suggest a novel mechanism of AMPK-dependent NO release in cardiac myocytes with putative pathophysiological relevance determined, at least in part, by its capability to reduce the extent of contractile dysfunction associated with hyperosmotic stress.

Keywords Hyperosmotic stress · Nitric oxide · AMPK · Contractile dysfunction

Introduction

The osmolarity of body fluids is tightly controlled under physiological conditions [20]. However, the volume of cells, including cardiomyocytes, can change under pathological conditions. During hyperglycaemia, hyperlipidaemia, diabetes, heat stress, and severe dehydration cells suffer hyperosmotic stress and undergo shrinkage [3, 12, 21, 34, 35]. In the myocardium, this change in cell volume has been shown to result in contractile dysfunction and cell death [13, 21, 30].

Nitric oxide (NO) is synthesized by the nitric oxide synthase (NOS) and has been defined as an important second messenger and a critical regulator of cardiac excitation and contraction coupling (ECC) [47]. Indeed, NO has been demonstrated to promote a positive or negative inotropic effect in a concentration-dependent manner [39, 40, 43].

In addition, NO impacts on the myocardial adaptation to ischemia–reperfusion [6, 18, 19, 23, 26, 40] and the hypertrophic response to transverse aortic constriction [46].

Several reports have demonstrated that both mechanical and hypotonic stresses can increase NO release in cardiac cells [14, 15, 38]. However, whether hyperosmotic stress is also able to promote NO release in cardiac ventricular myocytes has not been established. NO can be produced in an NOS-independent manner [31] or by the activity of three NOS isoforms: nNOS, eNOS, and iNOS [47]. Interestingly, AMP-activated protein kinase (AMPK) has been described as a mechanical-stress sensor [16, 24] able to phosphorylate and activate nNOS [14, 33] and eNOS [9] resulting in enhanced NO production. Whether AMPK is involved in osmotic stress-induced NO production has never been evaluated. Thus, the aim of the present study was to examine if

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hyperosmotic stress is associated with NO release in cardiac myocytes and if so, to determine if AMPK acts as the mechanotransducer that links hyperosmotic stress-induced membrane deformation to NOS activation.

Hyperosmotic stress causes a severe decrease in myocyte contraction associated with a decrease in resting cell length and in the amplitude of cell shortening [21]. Hypotonic stress also promotes cardiomyocyte contractile dysfunction and we have recently shown that NO released during hypotonic stress provides contractile support that attenuates the swelling-induced negative inotropic effect [15]. Thus, yet another objective of the present study was to determine the functional implications of NO on the hyperosmotic stress-induced negative inotropic response.

We show for the first time that hyperosmotic stress activates AMPK, which then phosphorylates nNOS and eNOS, resulting in NO release in adult rat cardiac myocytes. This NO, through a cGMP/PKG-dependent mechanism, provides inotropic support that reduces the impact of hyperosmotic stress-induced contractile dysfunction.

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.

Myocyte isolation

Male Wistar rats (200–300 g) were anaesthetised by an intraperitoneal injection of ketamine (79 mg/kg) and diazepam (5 mg/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 [15]. In brief, the hearts were mounted on a Langendorff perfusion apparatus and were perfused retrogradely at a constant flow with a HEPES 1 mM $CaCl_2$-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 of HEPES containing 0.1 mM EGTA, and then for 6 min with HEPES 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 crossed four steps of decantation and re-suspension in HEPES solutions with increasingly concentration of $CaCl_2$, until reaching a final concentration of 1 mM $CaCl_2$ where they were kept in suspension until their use.

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

Experimental protocol

Myocytes were field stimulated by external platinum electrodes at 0.5 Hz and superfused (1 ml/min at room temperature) with isotonic solution (IS: 309 mOsm) and then exposed to hypertonic solution (HS: 440 mOsm). For control experiments, cardiac myocytes were kept in IS throughout the experiment. These HEPES buffered solutions were prepared as follows:

IS (mM): NaCl 91, KCl 5.3, $MgCl_2$ 0.5, $CaCl_2$ 1, Glucose 10, HEPES 10, Manitol 91.5 pH 7.4 (with NaOH).

HS (mM): NaCl 91, KCl 5.3, $MgCl_2$ 0.5, $CaCl_2$ 1, Glucose 10, HEPES 10, Manitol 224.35 pH 7.4 (with NaOH).

The osmolarity of HS was chosen considering that similar values were used by others to study the impact of hypertonicity on contractile function and/or Ca^{2+} handling in different cardiac preparations [1, 27, 29, 45]. 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 [12, 37].

Measurement of cell volume

We acquired brightfield images of cells superfused with IS and HS to measure cell width and length. We calculated cell volume based on the formula: volume (pl) = $(\pi wdl)/4$, where *wdl* represents cell width × depth × length and the cell is assumed to be an elliptical cylinder with a cell depth of 1/3 cell width [8].

Measurement of intracellular nitric oxide

Cardiac myocytes were loaded with 5 µM DAF-FM diacetate (molecular probes) for 30 min, and imaged by epifluorescence on a Zeiss 410 inverted confocal microscope (LSM Tech, Pennsylvania, USA). Excitation at 488 nm was provided by an Argon laser and emission was collected in a range of 510–530 nm. Fluorescence was acquired for 2.5 s at 5 min intervals over the duration of 25 min in absence and presence of L-NAME (2.5 mM), nitroguanidine (240 nM), wortmannin (1 µM) and Dorsomorphin (10 µM). To minimize photobleaching, the lowest laser intensity possible was used.

Cell shortening measurement

We measured cell shortening by video detection of cell borders [15] in cardiac myocytes superfused with IS or HS in the absence or presence of L-NAME (2.5 mM), Dorsomorphin (10 µM), ODQ (10 µM), and KT5823 (1 µM). The acquired data were stored for further analysis using a suitable software (IonOptix analysis).

Immunodetection by Western blot analysis

Cardiac myocytes seeded onto 25 mm Petri dishes were maintained during 30 min in IS or HS in the absence or presence of 10 µM of Dorsomorphin. Then, cells were collected and homogenized with lysis buffer. Proteins were measured by the Bradford method using BSA as standard. Homogenates (~ 90 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 antibodies raised against anti-phospho-Ser1177 endothelial NO synthase (Cell Signaling, dilution 1:1000), anti-phospho-Ser1417 neuronal NO synthase (Abcam, dilution 1:2000), and anti-eNOS (Sigma-Aldrich, dilution 1:1000) and anti-nNOS (Santa Cruz, dilution 1:500) for normalization. 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 the Image J software (NIH).

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 are expressed as mean ± SEM. Differences were considered significant at $p \leq 0.05$.

Results

Hyperosmotic stress promotes NO release in isolated ventricular rat myocytes

Figure 1 shows the changes in cell dimensions and NO production (measured by DAF-FM fluorescence), induced by switching superfusion from an isotonic solution (IS) to a hypertonic solution (HS). Figure 1a shows brightfield images and overall results indicating that superfusing cardiac myocytes with HS for 20 min significantly decreases cell width, length, and hence cell volume. Control experiments indicate that maintaining cells in IS for an equivalent time frame does not affect these parameters. Figure 1b depicts typical fluorescence images of a cardiomyocyte loaded with DAF-FM showing that the NO-sensitive fluorescence increases when the cell is superfused with HS. The overall results show that in myocytes stimulated at 0.5 Hz, changing superfusion from IS to HS induces a gradual increase in DAF-FM fluorescence which becomes significant after 5 min. In contrast, cells maintained in IS do not show a significant increase in DAF-FM fluorescence. These results indicate that hyperosmotic stress-induced cell deformation promotes NO release in adult rat cardiac myocytes.

Next, we examined the source of NO. Given that iNOS is only expressed during the immune response [2]; we studied the contribution of nNOS and eNOS to hyperosmotic stress-induced NO production. Cardiac myocytes were superfused with HS either in the absence or in the continued presence of 2.5 mM of the non-selective NOS inhibitor, L-NAME (L-N), or 240 nM of the selective nNOS inhibitor, nitroguanidine (NTG) or 1 µM of the PI3K inhibitor, Wortmannin (WORT), which has been shown to inhibit Ca^{2+}-independent eNOS activation [25] or NTG plus WORT. Figure 2a shows that after 20 min of superfusion in the presence of these inhibitors, HS fails to increase NO production, suggesting that both nNOS and eNOS are involved. Figure 2b depicts data from experiments, showing that there was no difference in the degree of cell shrinkage when cells were superfused with HS in the presence of these inhibitors compared to HS alone. These results exclude the possibility that the failure to observe an increase in DAF-FM fluorescence with HS in the presence of inhibitors was due to the prevention of cellular shrinkage.

To confirm the participation of nNOS and eNOS in hyperosmotic stress-induced NO production we examined, by western blot, the activity of these enzymes. Figure 3 depicts typical western blots and overall results showing that maintaining cardiomyocytes in HS increases the phosphorylation of the activating sites of nNOS and eNOS, Ser1417, and Ser1177, respectively, compared to cardiomyocytes exposed to IS, suggesting that these two isoforms are activated during hyperosmotic stress.

AMP-dependent kinase (AMPK) mediates the activation of nNOS and eNOS during hyperosmotic stress

AMPK has been shown to be activated by mechanical stress in cardiac muscle [16, 24]. Moreover, AMPK activation has been shown to promote eNOS [9] and nNOS [14] phosphorylation leading to an increase in NO production. Thus, we evaluated if AMPK was involved in hyperosmotic stress-induced NO production. Typical fluorescence images and overall results depicted in Fig. 4a show that 10 µM of the specific AMPK inhibitor, Dorsomorphin (DORSO), is able to prevent the increase in

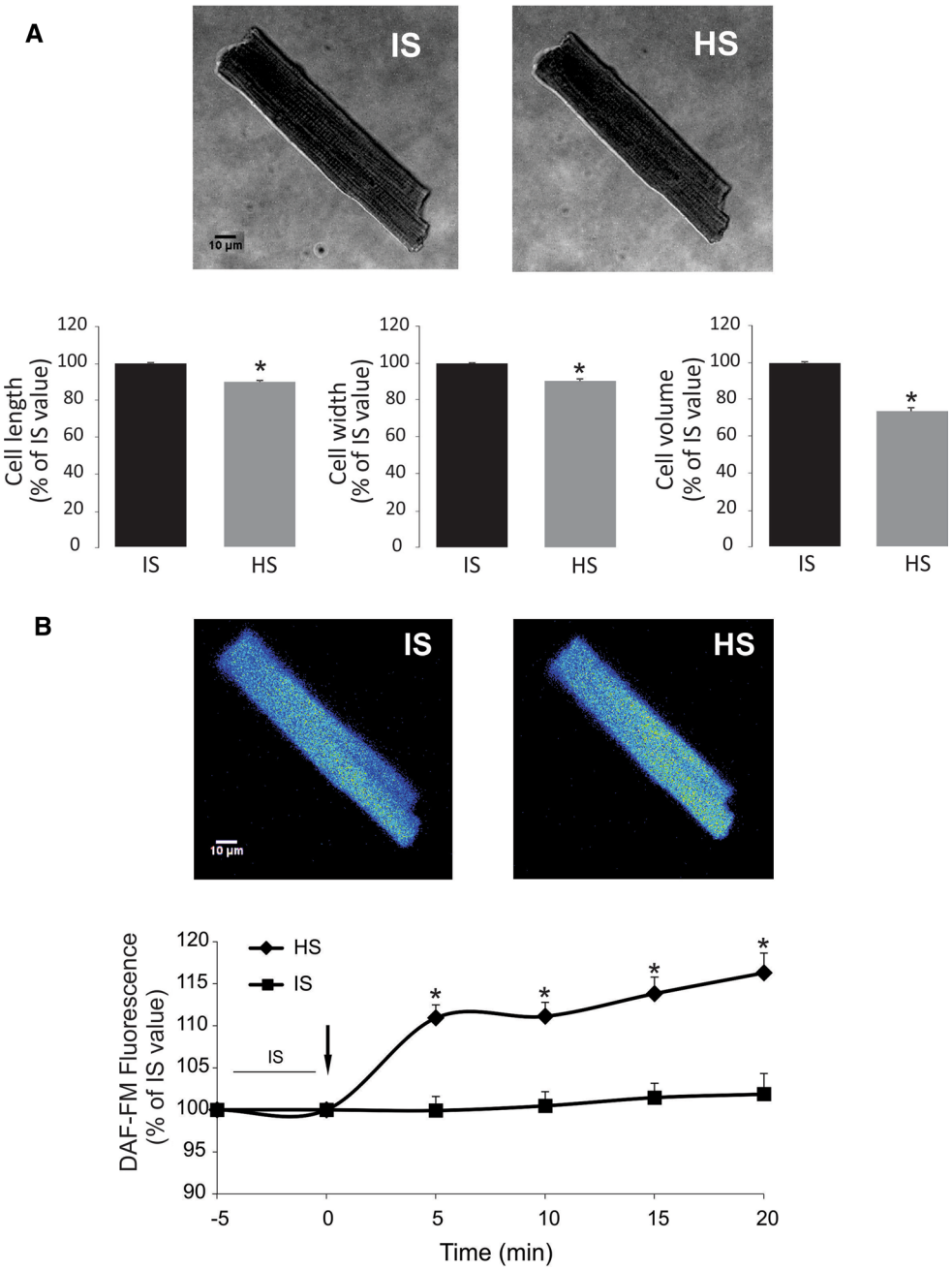

Fig. 1 Effect of hyperosmotic stress on cell dimensions and DAF-FM fluorescence in rat ventricular myocytes. **a** Representative brightfield images show a cardiomyocyte in IS and the same cardiomyocyte 20 min after superfusion with HS. The bar graph shows that superfusion with HS induced a significant decrease in cell length ($n = 24$ from 13 hearts), cell width ($n = 24$ from 13 hearts), and hence cell volume ($n = 24$ from 13 hearts) compared with cells maintained in IS ($n = 16$ from 10 hearts). Results are expressed as mean ± SEM; $*p \leq 0.05$ vs. cells maintained in IS. **b** Representative fluorescence images and overall results showing an increase in DAF-FM fluorescence that becomes significant 5 min after superfusion with HS ($n = 26$ from 16 hearts) compared to IS ($n = 16$ from 10 hearts). Arrow indicates where superfusion is changed from IS to HS or maintained in IS to serve as a control. Results are expressed as mean ± SEM; $*p \leq 0.05$ vs. cells maintained in IS

DAF-FM fluorescence observed in rat myocytes exposed to hyperosmotic stress (shown in Fig. 1b).

In addition, western blot analysis shows that, in the presence of DORSO, HS does not affect eNOS and nNOS phosphorylation compared to IS (Fig. 4b).

Taken together, these results suggest that this kinase is responsible for the activation of NOS isoforms and, consequently, NO production during hyperosmotic stress.

NO provides inotropic support during hyperosmotic stress

In adult cardiac myocytes, hyperosmotic stress has been shown to produce a negative inotropic effect [21]. Given that NO has been extensively shown to modulate cardiac contractility [43, 47], we investigated whether NO released during hyperosmotic stress also impacts on myocyte contractility.

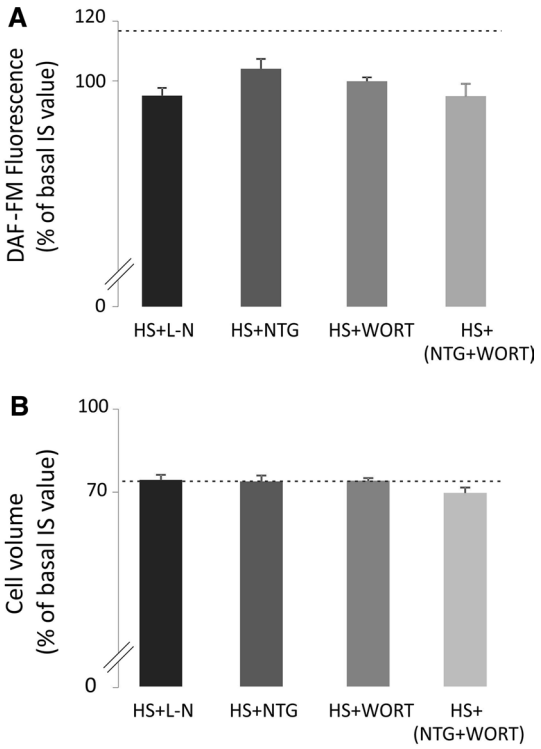

Fig. 2 Effect of pharmacological inhibition of NO production on DAF-FM fluorescence and cell volume in ventricular myocytes subjected to hyperosmotic stress. **a** Overall results showing the effect of 20 min of superfusion with HS in the presence of NOS inhibitors on DAF-FM fluorescence. In the presence of L-NAME (L-N $n = 10$ from 7 hearts), nitroguanidine (NTG $n = 10$ from 4 hearts), wortmannin (WORT $n = 16$ from 7 hearts), and NTG + WORT ($n = 6$ from 3 hearts). HS in the presence of NOS inhibitors failed to produce an increase in DAF-FM fluorescence compared to HS alone. The dotted line represents the mean fluorescence value of DAF-FM at 20 min of superfusion with HS alone (mean value shown in Fig. 1). Results are expressed as mean ± SEM. **b** Overall results showing the effect of 20 min of superfusion with HS in the presence of NOS inhibitors on cell volume. L-NAME (L-N $n = 10$ from 6 hearts), nitroguanidine (NTG $n = 10$ from 4 hearts), wortmannin (WORT $n = 16$ from 7 hearts), and NTG + WORT ($n = 6$ from 3 hearts) do not affect. The degree of cellular shrinkage compared with HS alone. The dotted line represents the mean value of cell volume after 20 min of superfusion with HS alone (mean value shown in Fig. 1). Results are expressed as mean ± SEM

Figure 5a depicts representative traces of twitch contractions and overall results of cardiomyocytes stimulated to contract at 0.5 Hz under isotonic conditions and 20 min after switching to HS, showing that superfusing cells with HS produces a pronounced negative inotropic effect (NIE). When similar experiments were performed in the continued presence 2.5 mM of L-NAME (L-N), to completely inhibit NO production, the HS-induced reduction in contractility was significantly exacerbated. Figure 5b depicts representative traces of twitch contractions and overall results showing that, similar to L-NAME, 10 µM of DORSO also exacerbates the HS-induced NIE. Taken together, these results suggest that NO produced by AMPK-dependent-NOS activation provides inotropic support during hyperosmotic stress.

Contractile support produced by NO is mediated by a cGMP/PKG-dependent mechanism

It has been well established that NO may modulate cardiac ECC both through cGMP-dependent and cGMP-independent mechanisms [47]. To explore the signaling cascade by which NO contributes to sustain contractile function during hyperosmotic stress, we performed experiments in the absence and presence of 10 µM of the guanylate cyclase inhibitor, ODQ.

Figure 6a depicts representative traces of twitch contractions and overall results of cardiomyocytes superfused with IS and HS in the presence of ODQ, showing that ODQ exacerbates the HS-induced NIE. Interestingly, these results are similar to those observed with L-NAME and DORSO (Fig. 5a, b), suggesting that the effect of NO on cardiomyocyte contraction is mediated by the cGMP signaling and not by cGMP-independent mechanisms.

In the cardiovascular system, cGMP exerts physiological actions through the activation of cGMP-dependent protein kinases (PKG) and/or through the activation or inhibition of phosphodiesterases [42]. To determine if cGMP was providing contractile support by a PKG-dependent or -independent mechanism, we examined the amplitude of contraction in cardiomyocytes superfused with IS and HS in the absence or presence of 1 µM of the specific PKG inhibitor, KT5823. Figure 6b shows the time course of cell shortening of cardiomyocytes superfused with IS or HS in the absence or presence of the PKG inhibitor. The average results show that superfusing cardiac myocytes for 25 min with IS either in the absence or presence of KT5823 does not change contraction amplitude compared to the basal value. On the other hand, PKG inhibition exacerbates the HS-induced NIE in a similar magnitude to that observed with ODQ, L-NAME, and DORSO, suggesting that the mechanism by which NO exerts contractile support is mediated by cGMP/PKG signaling.

Discussion

The present study shows, for the first time, that hyperosmotic stress promotes NO release in isolated ventricular rat myocytes. Indeed, NO production is mediated by AMPK-dependent nNOS (NOS1) and eNOS (NOS3) activation.

The results shown herein further show that NO released during this process has physiological impact given that it reduces hyperosmotic stress-induced negative inotropic effect via cGMP/PKG signaling.

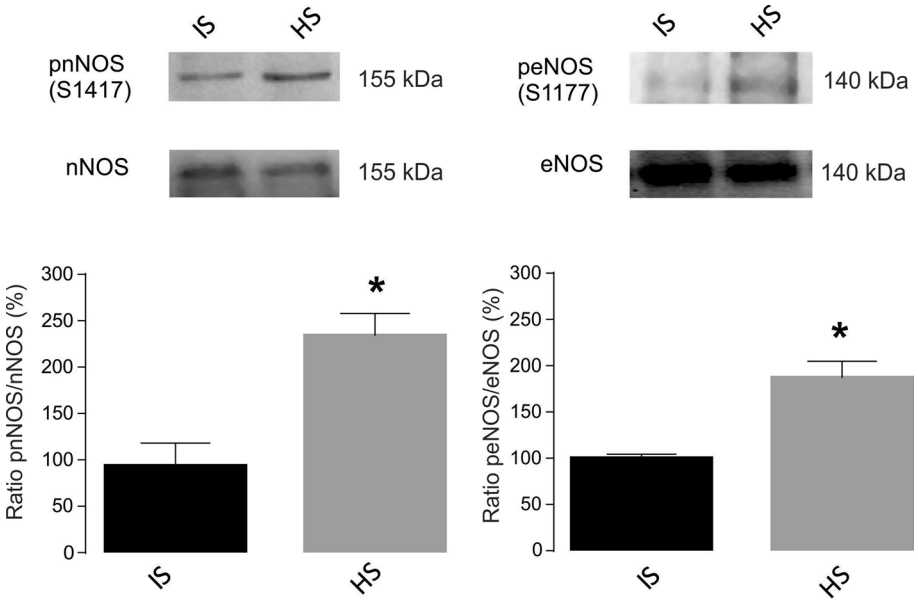

Fig. 3 Effect of HS on the activation of NOS isoforms. Representative blots and overall results showing that in cardiac myocytes, HS increases the phosphorylation of the activation sites of nNOS and eNOS compared to IS. Results are expressed as mean ± SEM from three independent experiments from three hearts; $^{*}p \leq 0.05$ vs. hearts perfused with IS

Hyperosmotic stress promotes NO production in rat ventricular cardiac myocytes

Several reports, including results from our laboratory, have shown that osmotic stress can promote NO release in cardiac and non-cardiac muscle [15, 25, 35, 41]. Although the previous studies have shown that exposing rats to water restriction can promote the activation of different NOS isoforms [34, 35], to date, there is no evidence that hyperosmotic stress can stimulate NO release at the cardiac myocyte level. In the present study, we show that superfusion of cardiac myocytes with HS decreases cell width, cell length, and hence cell volume, indicative of hypertonic cell shrinkage. This cellular shrinkage was associated with an increase in the fluorescence of the NO sensor, DAF-FM, which was not observed when cells were continuously superfused with IS (Fig. 1).

Confirming that the rise in DAF-FM fluorescence was due to an increase in NO production and not due to an increase in dye concentration after cell shrinkage, we observed that inhibiting NOS activity with L-NAME (L-N), nitroguanidine (NTG), wortmannin (WORT), and NTG + WORT prevented the HS-induced increase in DAF-FM fluorescence without affecting cell shrinkage (Fig. 2a, b). The same consideration allows us to exclude other limitations in the use DAF-FM as an NO sensor, such as the reported influence of oxygen reactive species [22] or pH on its emission [28, 44]. Taken together, we were able to detect an NOS-dependent increase in DAF-FM signal in cardiac myocytes upon hyperosmotic stress.

nNOS and eNOS mediate hyperosmotic stress-induced NO production

There are three known isoforms of NOS within the myocardium: nNOS and eNOS are constitutively expressed and require Ca^{2+} for their activation. The other isoform, iNOS, is mainly expressed during inflammatory processes [47]. To determine which NOS isoform was involved in hyperosmotic stress-induced NO release, we used (1) the non-specific NOS inhibitor, L-NAME (L-N); (2) the nNOS-specific inhibitor, nitroguanidine (NTG); (3) the PI3K inhibitor, Wortmannin (WORT) to inhibit eNOS [25] and NTG plus WORT to inhibit simultaneously nNOS and eNOS.

As shown in Fig. 2a, all NOS inhibitors significantly reduced HS-induced DAF-FM fluorescence. Interestingly, despite not reaching statistical significance, DAF-FM fluorescence in the context of global NOS inhibition (L-NAME or NTG +WORT), tended to be lower than the fluorescence in the presence of specific NOS isoform inhibition (NTG or WORT, alone), suggesting that both isoforms could be involved in NO production during hyperosmotic stress.

We studied cell volume in the presence of NOS inhibitors after 20 min of superfusion with HS to verify that NO production was blocked due to specific enzymatic inhibition and not to an unspecific effect of NOS inhibitors that could reduce the degree of cell shrinkage in response to HS. Indeed, all treatments were able to prevent the increase in DAF-FM fluorescence without significantly affecting the degree of cell shrinkage (Fig. 2).

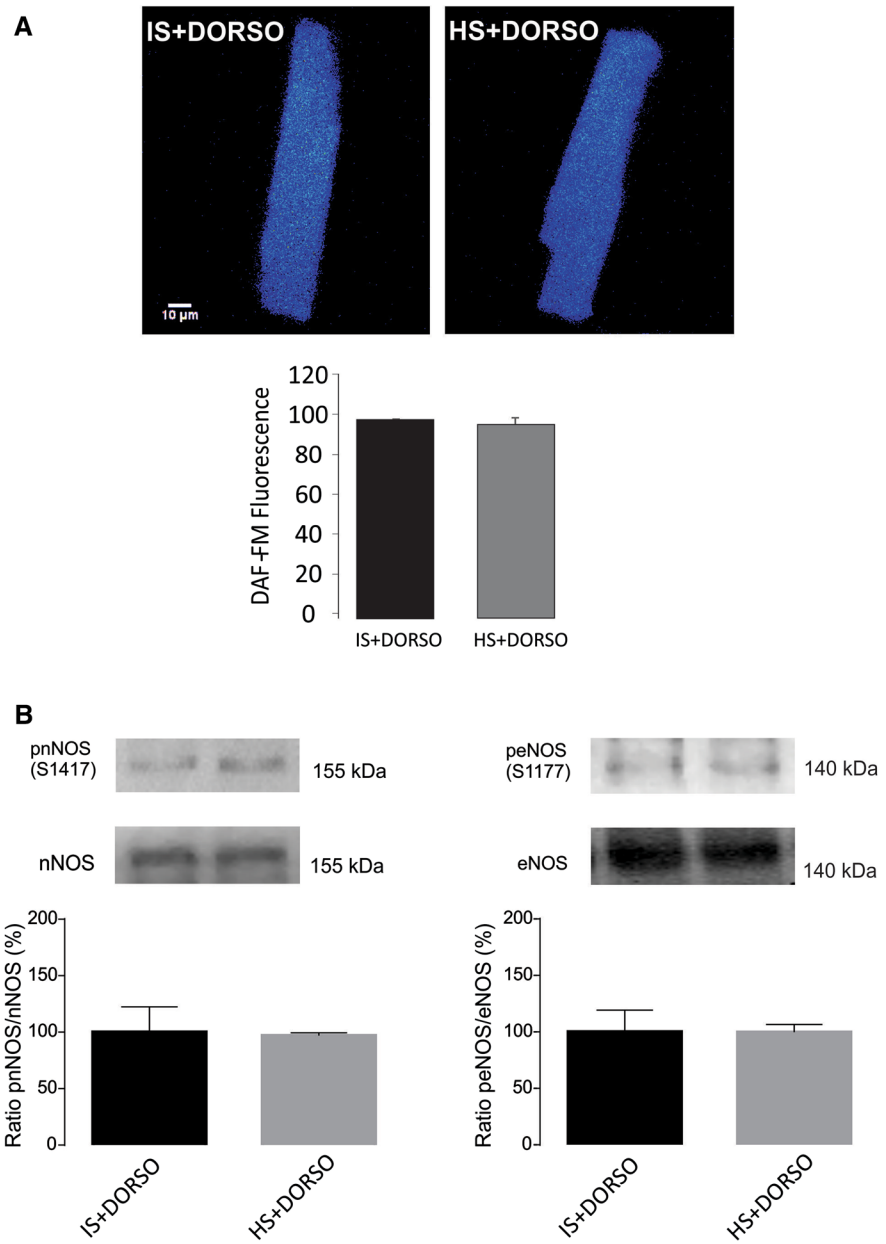

Fig. 4 Role of AMP-dependent kinase (AMPK) on NO production during hyperosmotic stress. **a** Representative fluorescence images and overall results at 20 min of cell superfusion with IS or HS in the presence of Dorsomorphin (DORSO), showing that the increase in DAF-FM fluorescence observed with HS alone (Fig. 1) is prevented. IS + DORSO and HS + DORSO $n = 18$ from 5 hearts. Results are expressed as mean ± SEM. **b** Representative blots and overall results, showing that in the presence of DORSO, HS does not affect eNOS and nNOS phosphorylation compared to IS. Results are expressed as mean ± SEM from three independent experiments from three hearts

The nNOS and eNOS enzymatic activity is modulated by phosphorylation at different sites leading to activation or inactivation of the enzyme [7, 9, 14, 17, 32]. It has been well defined that phosphorylation of sites Ser1417 and Ser1177 of nNOS and eNOS, respectively, promotes enzyme activation leading to NO production [5, 36]. Confirming the involvement of nNOS and eNOS in hyperosmotic stress-induced NO production, we showed, by western blot analysis, that incubation of cardiac myocytes with HS enhances the phosphorylation of these sites and, therefore, enzyme activation (Fig. 3) compared to IS. Taken together, these results suggest that nNOS and eNOS are the isoforms responsible in NO release during hyperosmotic stress.

We have previously reported that cardiac myocyte axial stretch promotes NO release and that the isoform involved is eNOS [38]. More recently, we demonstrated that hypotonic stress causes an increase in the dimension of the cell in the radial direction in the absence of changes in axial length. In this case, cell swelling was associated with an increase in NO production mediated primarily by the activation of nNOS [15]. In the present study, we showed that hyperosmotic stress, which promotes the deformation of the cardiac myocyte both in the axial and radial direction, is associated with both nNOS and eNOS activation. Considering these results, it is tempting to speculate that NOS enzymes are able to decode membrane deformation becoming deferentially activated depending on the direction in which the cell

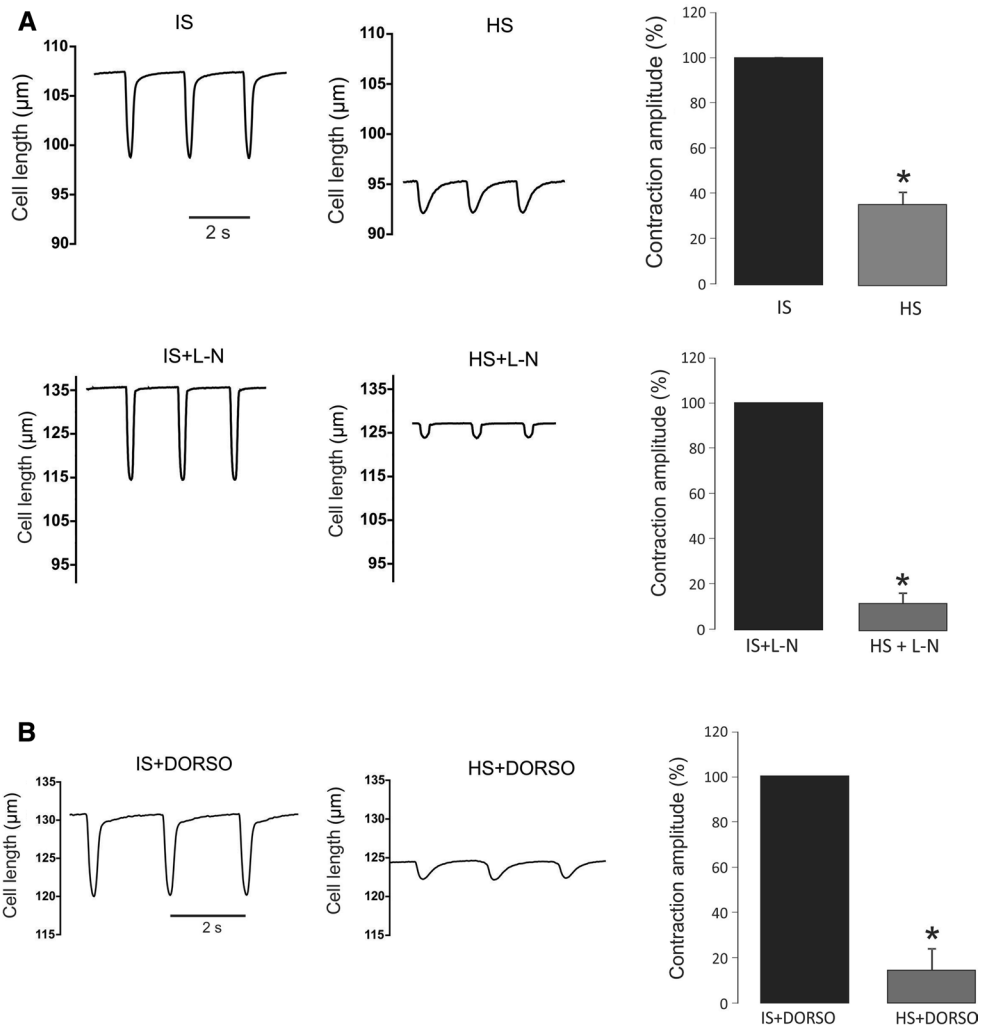

Fig. 5 Inhibiting NO production exacerbates the HS-induced negative inotropic effect. **a** Typical tracings of cell length and overall results of contraction amplitude of cells superfused with IS and after 20 min superfusion with HS, in the absence or presence of the NOS inhibitor (L-NAME: L-N). HS produces a negative inotropic effect and this effect is significantly enhanced by L-N. IS and HS alone $n = 16$ from 12 hearts; IS + L-N and HS + L-N $n = 11$ from 5 hearts. Results are expressed as mean ± SEM; $^*p \leq 0.05$ vs. cells maintained in IS or IS + L-N. **b** Typical tracings of cell length and overall results of contraction amplitude of cardiomyocytes superfused with IS and after 20 min superfusion with HS, both in presence of Dorsomorphin (DORSO), showing that DORSO also exacerbates de HS-induced negative inotropic effect. IS + DORSO and HS + DORSO $n = 12$ from 5 hearts. Results are expressed as mean ± SEM; $^*p \leq 0.05$ vs. cells maintained in IS + DORSO

deforms. Experiments are currently underway, in our laboratory, to test this hypothesis.

AMP-dependent kinase (AMPK) mediates the activation of nNOS and eNOS during hyperosmotic stress

It has been recently described that the cytoskeleton and the AMP-dependent kinase (AMPK) can interact directly in cardiac muscle [10]. Moreover, it has been reported that mechanical stress can activate this kinase [16] and that AMPK-dependent phosphorylation of nNOS [14] and eNOS [9] promotes NO release in cardiac muscle. Thus, we evaluated whether this kinase was responsible for the activation of NOS isoforms during hyperosmotic stress-induced membrane deformation. As can be observed in Fig. 4a, the specific AMPK inhibitor, Dorsomorphin (DORSO), was able to prevent the increase in DAF-FM fluorescence produced by superfusing cells with HS. These results, together with those showing that DORSO also prevents the increase in the level of phosphorylation of Ser1417 and Ser1177 of nNOS and eNOS, respectively (Fig. 4b), provide substantial evidence supporting the contention that AMPK acts as a mechanotransducer linking hyperosmotic stress-induced membrane deformation to NOS activation and NO production in adult cardiac myocytes.

NO provides inotropic support during hyperosmotic stress

In adult cardiac myocytes, hyperosmotic stress has been shown to produce a negative inotropic effect due to, at least in part, a decrease in Ca^{2+} myofilament sensitivity [21]. Consistently, our results show that HS produces a pronounced reduction in cardiomyocyte contraction amplitude (Fig. 5).

Given that NO has been extensively shown to modulate cardiac contractility [40, 43, 47] and that recent results from our laboratory demonstrate that nNOS-dependent NO release contributes to sustain cardiomyocyte contractile function during hypotonic stress [15], we investigated

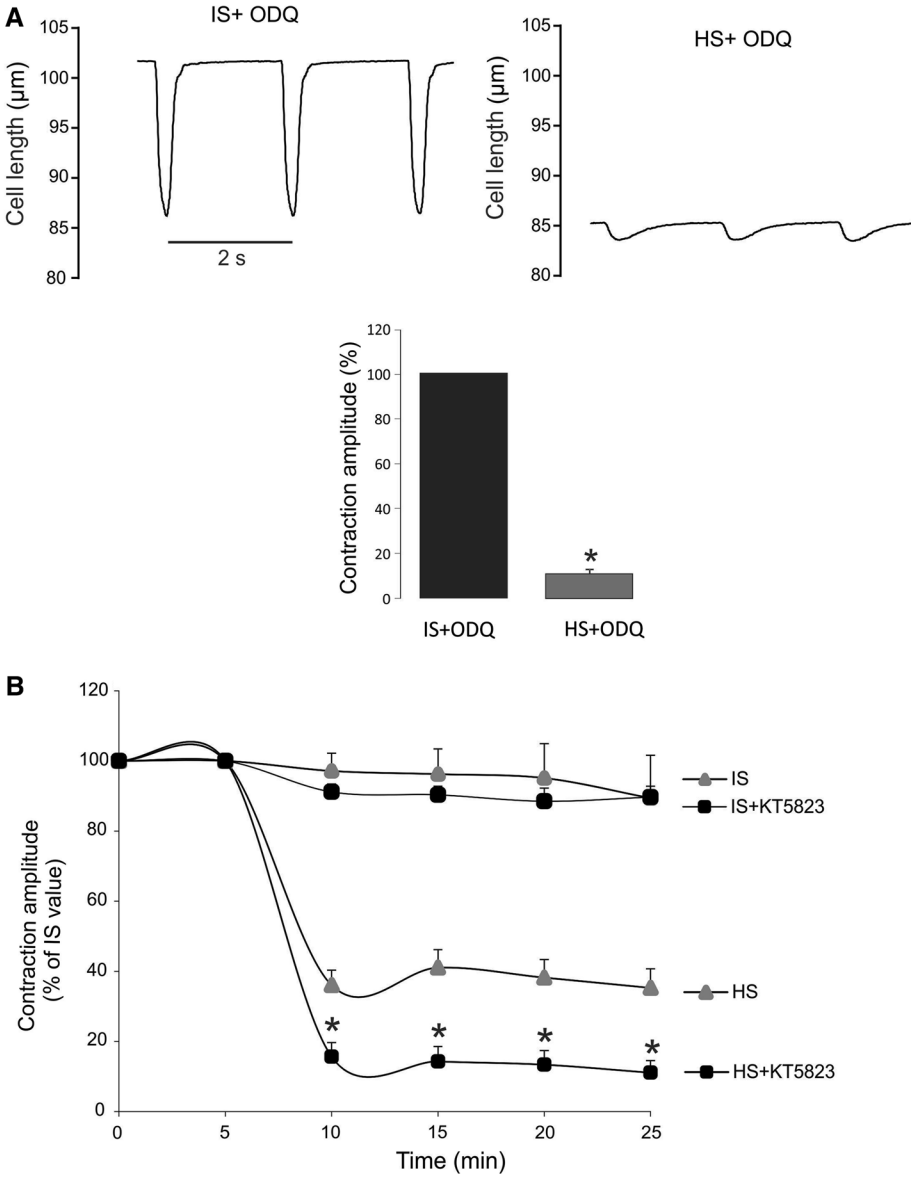

Fig. 6 cGMP/PKG signaling mediates NO-dependent contractile support. **a** Typical tracings of cell length and overall results of contraction amplitude of cardiomyocytes superfused with IS and after 20 min of superfusion with HS, both in presence of the guanylate cyclase inhibitor (ODQ), showing that ODQ exacerbates de HS-induced negative inotropic effect. IS + ODQ and HS + ODQ $n = 8$ from 2 hearts. IS and HS were supplemented with DMSO to control for the vehicle used in the preparation of IS + ODQ and HS + ODQ. Results are expressed as mean ± SEM; $^*p \leq 0.05$ vs. cells maintained in IS + ODQ. **b** Time course of the effect of hyperosmotic stress on cell shortening of myocytes superfused with IS and HS in the absence or presence of the PKG inhibitor (KT5823). KT5823 does not affect the amplitude of cell shortening in cells superfused with IS but it exacerbates de HS-induced negative inotropic effect. IS $n = 4$ from 3 hearts, HS $n = 16$ from 12 hearts, and IS + KT5823 $n = 7$ from 3 hearts, HS + KT5823 $n = 13$ from 3 hearts. IS and HS were supplemented with DMSO to control for the vehicle used in the preparation of IS + KT5823 and HS + KT5823. Results are expressed as mean ± SEM; $^*p \leq 0.05$ vs. cells maintained in HS

whether NO released during hyperosmotic stress also impacts on cardiomyocyte contractility.

Here, we show that inhibiting global NO production with the NOS inhibitor, L-NAME, or preventing NO release by inhibiting the activation of NOS isoforms with DORSO (Fig. 5) enhances the HS-induced negative inotropic effect. Although our results do not shed mechanistic insight into the mechanism that determines the hyperosmotic stress-induced negative inotropic effect, the results presented herein suggest that NO provides a mechanism of contractile support that prevents an even greater reduction in contractility produced by cell shrinkage.

It is well established that NO may modulate cardiac ECC both through cGMP-dependent and cGMP-independent mechanisms [47]. Our results showing that guanylate cyclase inhibition with ODQ (Fig. 6a) mimics the effect of inhibiting NOS suggest that cGMP-dependent mechanisms mediate the NO-dependent contractile support observed during hyperosmotic stress. Moreover, we studied the contractile amplitude of cardiac myocytes exposed to IS or HS in absence and continued presence of the PKG inhibitor, KT5823. As it can be seen in the time course of contraction amplitude (Fig. 6b), the superfusion of cardiomyocytes with IS in the absence or presence of the PKG inhibitor did not affect the contraction amplitude. Nevertheless, the superfusion with HS + KT5823 enhanced the HS-induced negative inotropic effect with the same pattern as in presence of L-NAME and DORSO (Fig. 5). Thus, these results suggest that NO, through a cGMP/PKG-dependent mechanism, would contribute to sustain myocyte contractility during hyperosmotic stress, preventing an even larger reduction in contractile function.

In a previous study, we showed that during hypotonic stress, nNOS-dependent NO release provides contractile support by increasing the Ca^{2+} transient through a cGMP/PKG-dependent increase in SR Ca^{2+} release [15]. It is tempting to speculate that during hyperosmotic stress, a similar mechanism could be responsible for preventing a larger decrease in contractility. However, identifying the mechanism underlying the NO-dependent contractile support was beyond the scope of this study. Interestingly, different to hypotonic swelling, which only activates nNOS [15], we show herein that hyperosmotic stress activates both NOS isoforms, eNOS and nNOS. We hypothesize that during this type of osmotic stress, NO produced by nNOS, which is localized mainly at the SR and has facilitative effects on contractility [4, 15], would be playing a protective role by sustaining contractility, while NO produced by eNOS which is targeted to the sarcolemma and caveolae [11] could be involved in other, yet undefined physiological functions such as cell survival or growth. Experiments are currently underway in our laboratory to test this hypothesis.

In summary, our results show, for the first time, that hyperosmotic stress promotes NO release mediated by AMPK-dependent nNOS and eNOS activation. We show that this NO impacts on hyperosmotic stress-induced contractile dysfunction by providing inotropic support through a cGMP/PKG-dependent mechanism. This mechanism could be important to sustain contractile function in pathological situations that are associated with hyperosmotic stress and depressed contractility. Our results also suggest that contractile dysfunction occurring as a result of hyperosmotic stress could be exacerbated in pathological states associated with a low NO availability.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interest.

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