

Hypotonic swelling promotes nitric oxide release in cardiac ventricular myocytes: impact on swelling-induced negative inotropic effect

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Aims	Cardiomyocyte swelling occurs in multiple pathological situations and has been associated with contractile dysfunction, cell death, and enhanced propensity to arrhythmias. We investigate whether hypotonic swelling promotes nitric oxide (NO) release in cardiomyocytes, and whether it impacts on swelling-induced contractile dysfunction.
Methods and results	Superfusing rat cardiomyocytes with a hypotonic solution (HS; 217 mOsm), increased cell volume, reduced myocyte contraction and Ca^{2+} transient, and increased NO-sensitive 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM) fluorescence. When cells were exposed to HS + 2.5 mM of the NO synthase inhibitor L-NAME, cell swelling occurred in the absence of NO release. Swelling-induced NO release was also prevented by the nitric oxide synthase 1 (NOS1) inhibitor, nitroguanine, and significantly reduced in NOS1 knockout mice. Additionally, colchicine (inhibitor of microtubule polymerization) prevented the increase in DAF-FM fluorescence induced by HS, indicating that microtubule integrity is necessary for swelling-induced NO release. The swelling-induced negative inotropic effect was exacerbated in the presence of either L-NAME, nitroguanine, the guanylate cyclase inhibitor, ODQ, or the PKG inhibitor, KT5823, suggesting that NOS1-derived NO provides contractile support via a cGMP/PKG-dependent mechanism. Indeed, ODQ reduced Ca^{2+} wave velocity and both ODQ and KT5823 reduced the HS-induced increment in ryanodine receptor (RyR2, Ser2808) phosphorylation, suggesting that in this context, cGMP/PKG may contribute to preserve contractile function by enhancing sarcoplasmic reticulum Ca^{2+} release.
Conclusions	Our findings suggest a novel mechanism for NO release in cardiomyocytes with putative pathophysiological relevance determined, at least in part, by its capability to reduce the extent of contractile dysfunction associated with hypotonic swelling.
Keywords	Hypotonic swelling • Nitric oxide • Contractile dysfunction • Ischaemia reperfusion

1. Introduction

Tissue osmolarity is tightly regulated under physiological conditions.¹ However, under diverse pathological situations, cells undergo hypotonic swelling^{2–4} that dilutes intracellular components and deforms cellular membranes and the underlying cytoskeleton. This results in changes in the activity of signalling molecules and ion channels leading to alterations in cellular function. In the heart, cell swelling occurs in diabetic or septic shock and elective cardioplegia, and is particularly important in the context

of ischaemia and reperfusion (I/R) where it results in structural, ionic, and electrical remodelling that have been shown to contribute to contractile dysfunction, cell death, and enhanced propensity for arrhythmias.⁴

Nitric oxide (NO), synthesized by the nitric oxide synthase (NOS), has been defined as a second messenger and a regulator of cardiac excitation and contraction coupling (ECC).⁵

Cardiomyocyte axial stretch has been shown to promote NO release,⁶ and recent evidence has emerged showing that hypotonic swelling can promote NO release in endothelial cells and in outer hair cells of

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the cochlea.^{7,8} However, whether hypotonic swelling can promote NO release in cardiac ventricular myocytes has not been established. Thus, the present study was designed to examine whether cardiomyocyte hypotonic swelling is associated with NO release and if so, to determine its functional implications on the swelling-induced negative inotropic response.

Using an NO-sensitive fluorescent dye, we show for the first time that hypotonic swelling-induced cytoskeleton deformation leads to NOS1-dependent NO release in cardiomyocytes. This NO, through a cGMP/PKG-dependent mechanism, provides inotropic support that could reduce the impact of hypotonic swelling-induced contractile dysfunction.

2. 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.

2.1 Myocyte isolation

Male Wistar rats (200–300 g) were anaesthetised by an intraperitoneal injection of sodium pentobarbitone (35 mg/kg body weight) and hearts were excised when plane three of phase III of anaesthesia was reached. Plane three of phase III of anaesthesia was verified by the presence of slow deep diaphragmatic breathing, loss of the corneal reflex, and the absence of tongue retraction.

Cardiomyocytes were isolated by collagenase-based enzymatic digestion using a previously described technique.⁹

Using a similar protocol, myocytes were isolated from 3-month-old C57BL/6 (WT, Jackson Laboratories, Bar Harbor, ME, USA) and transgenic mice with homozygous deletions for NOS1 bred on a C57BL/6 background (NOS1KO, Jackson Laboratories).

2.2 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) during 5 min and then exposed to hypotonic solution (HS, 217 mOsm). For control experiments, cardiomyocytes were kept in IS throughout the experiment. These HEPES-buffered solutions were prepared as previously described by others.¹⁰ In brief: HS (in mmol/L): NaCl 91, KCl 5.3, MgCl₂ 0.5, CaCl₂ 1, glucose 10, and HEPES 10, titrated to pH 7.4; IS: HS + mannitol 91.5 mM.

2.3 Measurement of intracellular NO and cell width in cardiomyocytes

Cardiomyocytes were loaded with 5 μ M 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM) diacetate (Molecular Probes) for 30 min, and imaged by epifluorescence on a Zeiss 410 inverted confocal microscope (LSM Tech, Wellsville, PA, 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. To minimize photobleaching, the lowest possible laser intensity was used. In parallel, we acquired brightfield images to measure cell width which was used as an estimation of the degree of cell swelling.¹⁰

2.4 Cell membrane and T-tubular system imaging

After 20 min treatment with IS or HS, cells were loaded with di-8-aminonaphthylethylpyridinium (Di-8-ANNEPS, 5 μ M; Molecular Probes) for 2 min.¹¹ Fluorescent images were acquired by exciting with an Argon laser at 488 nm. Emission was collected at 560 nm.

In images from Di-8-ANNEPS-stained myocytes, we performed quantitative analysis of T-tubular (TT) architecture using the *Auto TT analysis program*, kindly provided by Dr Long-Sheng Song, Iowa University.¹²

2.5 Calcium transient and cell shortening measurement

Myocytes were loaded with 10 μ M fura-2-acetoxymethyl ester during 12 min. Fura-2 fluorescence was measured on an inverted microscope adapted for epifluorescence by an IonOptix 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_i²⁺.⁹

The amplitude of the Ca²⁺ transient and the integral of the Na⁺/Ca²⁺ exchanger (NCX) inward current induced by rapid application of 15 mM caffeine were used to estimate sarcoplasmic reticulum (SR) Ca²⁺ content.^{9,13} The NCX current was measured with an Axoclamp 2A amplifier using the whole-cell voltage-clamp technique. Pipette solution contained (in mM): K-aspartate 120, KCl 20, Na₂ATP 4, MgCl₂ 4, HEPES 10, glucose 10, and GTP 0.1; titrated to pH 7.2. All experiments were performed at 30°C using IS or HS supplemented with 0.1 mM ClBa. To ensure that the SR was consistently loaded with Ca²⁺, caffeine-dependent currents we measured after a 1-min rest at a holding potential of –80 mV.

2.6 Detection of spontaneous Ca²⁺ release by confocal microscopy

Myocytes were loaded with 10 μ M fluo-3-acetoxymethyl ester and mounted on a Zeiss 410 inverted confocal microscope (LSM Tech). Cells were imaged in a linescan mode along their long axis, exciting with the 488 nm line of an argon laser and collecting emission at >515 nm.⁹ Data were analysed using the 'Sparkmaster' plugin for ImageJ.¹⁴

2.7 Immunodetection by western blot analysis

Samples of isolated myocytes with a 70 \pm 10% yield of viable cells were subjected to control isotonic or hypotonic protocols in the presence or absence of 10 μ M of the guanylate cyclase inhibitor, ODQ, or 1 μ M of the PKG inhibitor, KT5823. Twenty minutes after treatment with IS/IS or IS/HS, myocytes were collected and lysed with ice-cold lysis buffer for 30 min.

Proteins from myocyte suspensions were measured by the Bradford method using BSA as standard. Lysates (~90 μ g 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 phospho-Ser2808-RyR2 (Badrilla, Leeds, UK) and anti-GAPDH (Millipore, Billerica, MA, USA) 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 ImageJ software (NIH).

2.8 Statistical analysis

Unpaired Student's *t*-test and one-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 means \pm SEM. Differences were considered significant at *P* \leq 0.05.

3. Results

3.1 Hypotonic swelling promotes NO release in isolated ventricular rat myocytes

The effect of switching superfusion from an IS to a HS on cell width and DAF-FM fluorescence is depicted in Figure 1. Figure 1A shows typical images indicating that after 20 min superfusion with HS cells maintain a well-organized sarcomere structure. Brightfield images also indicate

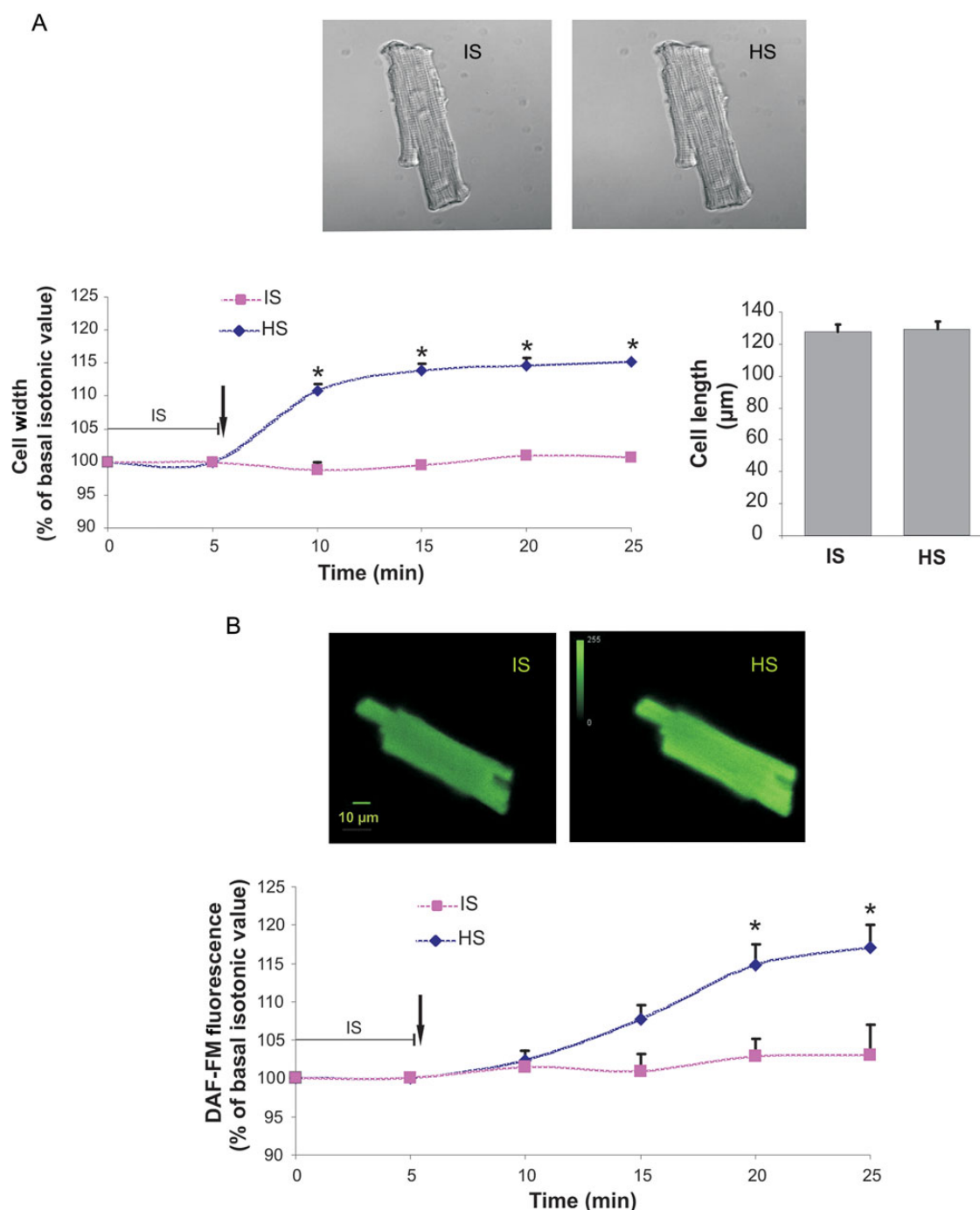


Figure 1 Effect of hypotonic superfusion on cell width and DAF-FM fluorescence in rat ventricular myocytes. (A) Representative brightfield images show a myocyte in IS and 20 min after superfusion with HS. Overall results below show that superfusion with HS induced a significant increase in cell width ($n = 27$ cells from 20 hearts) and had no effect on cell length ($n = 24$ cells from 19 hearts) compared with cells maintained in IS ($n = 11$ cells from 8 hearts). (B) Representative fluorescence images and overall results ($n = 35$ cells from 24 hearts) showing a gradual increase in DAF-FM fluorescence that becomes significant 15 min after superfusion with a HS. Arrows indicate where superfusion is changed from IS to HS or maintained in IS to serve as control. Results are expressed as mean \pm SEM; * $P < 0.05$ vs. cells in IS.

that HS noticeably enhances cell width. In contrast, cell length was not significantly enhanced, indicating that HS produces significant cell swelling without axial stretch. Control experiments show that maintaining cells in IS for an equivalent time frame does not affect cell width. Consistent with other reports,¹⁰ there was no evidence of regulatory volume

decrease (RVD) throughout the duration of the experiment. Figure 1B depicts typical images of a myocyte 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 15 min, reaching a plateau after 20 min. Similar results were obtained when DAF-FM fluorescence was monitored in quiescent myocytes (not shown). These results indicate that hypotonic swelling promotes NO release in adult rat cardiomyocytes. Interestingly, the time course of the increase in cell width is faster than that of DAF-FM fluorescence, being the time to half-maximum effect 4.42 ± 0.39 min for the change in cell width and 6.96 ± 0.67 min for DAF-FM fluorescence.

To identify the source of NO production, myocytes were superfused with HS in the absence or in the continued presence of 2.5 mM of the non-selective NOS inhibitor, L-NAME, 240 nM of the NOS1 selective inhibitor, nitroguanidine (NTG), or 1 μ M of the PI3K inhibitor, Wortmannin, which has been shown to inhibit Ca^{2+} -independent NOS3 activation.⁷ Figure 2A shows that HS in the presence of these inhibitors produced a similar degree of cell swelling compared with HS alone. However, L-NAME and NTG completely inhibit a HS-induced increase in DAF-FM fluorescence. In contrast, Wortmannin fails to prevent NO release (Figure 2B). These results suggest that NOS1 is the source of swelling-induced NO release in cardiomyocytes. To confirm this, DAF-FM fluorescence was monitored in cells pretreated with Ryanodine (RY) plus cyclopiazonic acid (CPA), condition that has been shown to inhibit SR function

and the therein located NOS1.¹⁵ Figure 2C depicts overall results, showing that HS fails to increase DAF-FM fluorescence in the absence of a functional SR. To further confirm the involvement of NOS1, by a non-pharmacological approach, we measured DAF-FM fluorescence in myocytes isolated from NOS1 knockout (KO) mice and in their wild-type (WT) controls. Figure 2D shows that superfusion with HS produced a significant increase in DAF-FM fluorescence in WT mice myocytes, and that this increase was significantly reduced in NOS1KO myocytes. Although not attaining statistical significance, NOS1KO myocytes treated with HS showed a tendency to have higher DAF-FM fluorescence compared with NOS1KO myocytes treated with IS. These results suggest that NOS1 is the main source for NO production under hypotonic swelling. However, a minor contribution by other NOS isoforms could be expected.

3.2 Cytoskeleton integrity is required for hypotonic swelling-induced NO release

Given the deformation of cell structure promoted by osmotic water intake, we studied the impact of HS superfusion on cell membrane and TT architecture. Figure 3A shows typical fluorescence images and average results obtained in cells labelled with the lipophilic dye

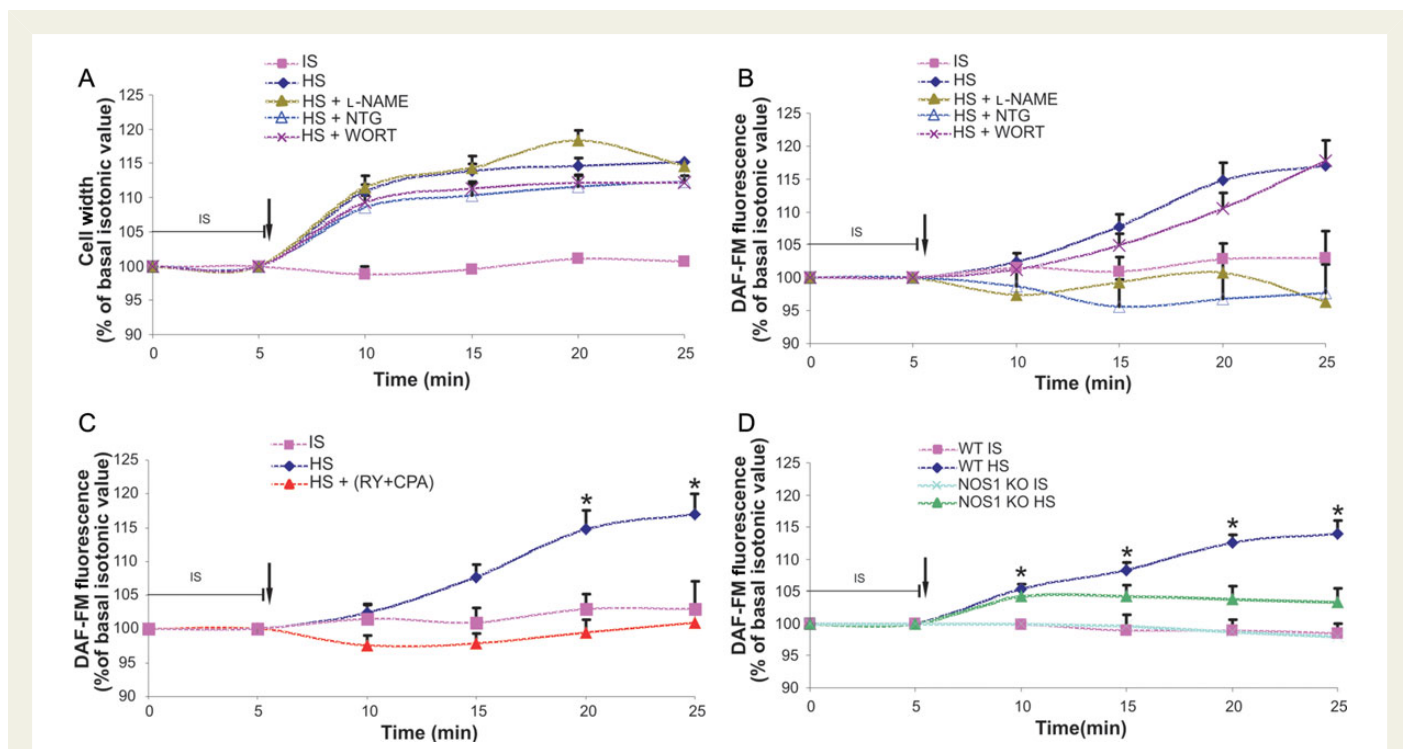


Figure 2 Effect of pharmacological inhibition and genetic deletion of NOS1 on cell width and DAF-FM fluorescence in ventricular myocytes subjected to hypotonic swelling. (A and B) Overall results showing the effect of hypotonic superfusion, either in the absence or presence of NOS inhibitors, on cell width and DAF-FM fluorescence. L-NAME ($n = 12$ cells from 7 hearts), Wortmannin (WORT; $n = 16$ from 7 hearts), and NTG ($n = 6$ from 4 hearts) did not significantly affect the degree of cell swelling. However, whereas cells treated with WORT showed a significant increase in DAF-FM fluorescence similar to the one observed in cells treated with HS alone, cells treated with L-NAME or NTG showed no change in fluorescence when superfused with HS. Results are expressed as mean \pm SEM; * $P < 0.05$ vs. cells in IS. Arrows indicate where superfusion is changed from IS to HS (except in cells maintained in IS). (C) Overall results showing that inhibiting SR function with Ryanodine (Ry) plus CPA prevents the increase in DAF-FM fluorescence induced by superfusion with HS ($n = 11$ cell from 6 hearts). Arrow indicates where superfusion is changed from IS to HS (except in cells maintained in IS). Results are expressed as mean \pm SEM; * $P < 0.05$ vs. cells in IS. (D) Overall results showing that superfusion with HS significantly increases DAF-FM fluorescence in WT mice myocytes ($n = 4$ IS and $n = 8$ HS from 3 hearts). Whereas this increase is reduced in myocytes from NOS1 KO mice ($n = 4$ IS and $n = 12$ HS from 3 hearts). Arrow indicates where superfusion is changed from IS to HS (except in cells maintained in IS). Results are expressed as mean \pm SEM; * $P < 0.05$ vs. cells in IS.

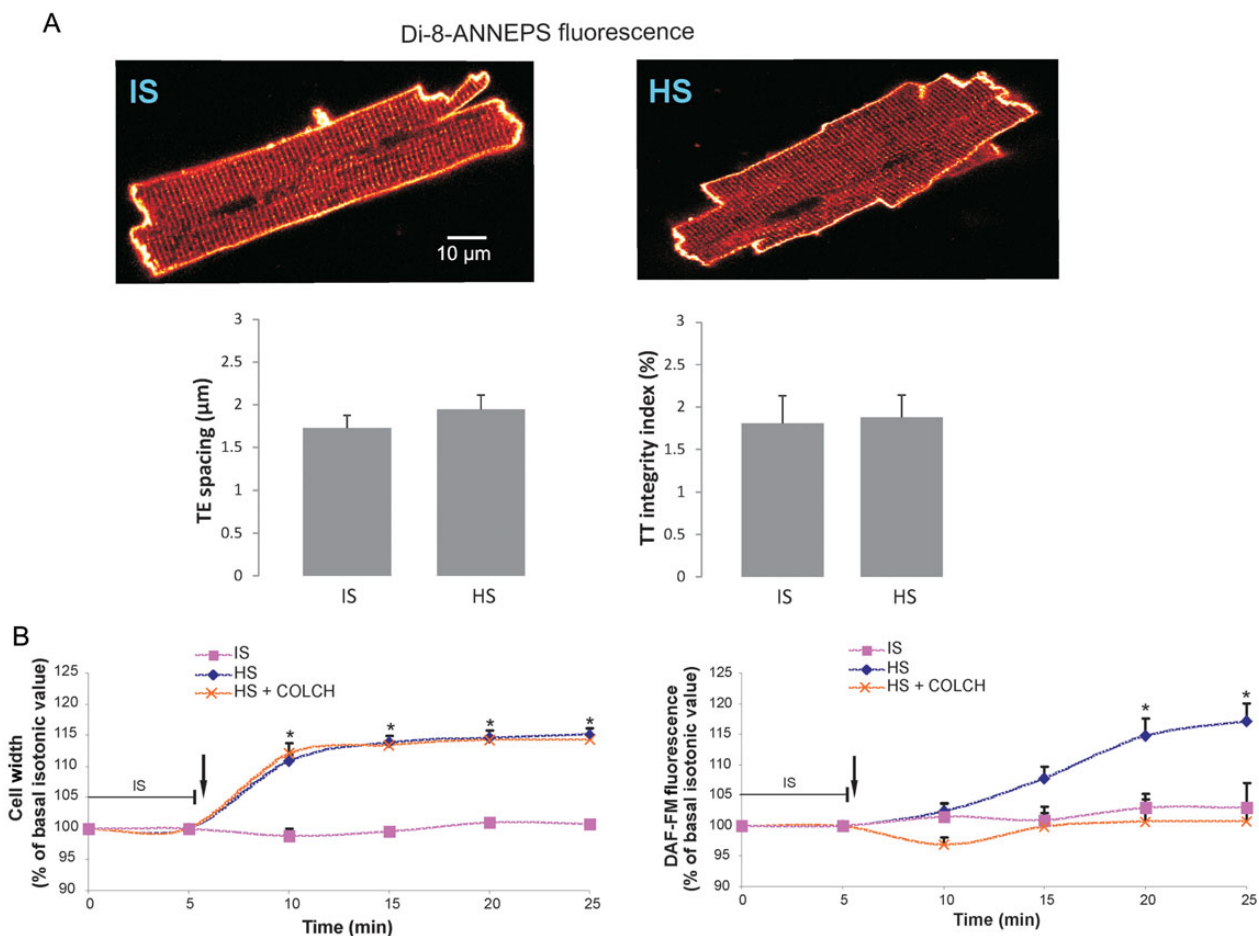


Figure 3 Role of membrane TT system and cytoskeleton in hypotonic swelling-induced signal transduction. (A) Representative images and overall results showing the effect of hypotonic superfusion on cell membrane and TT system integrity. After 20 min in either IS or HS, ventricular myocytes were loaded with the voltage-sensitive indicator Di-8-ANNEPS. T-tubules are identified as TEs. Cells treated with HS maintain sarcolemmal and TT integrity ($n = 21$ cells from 7 hearts). (B) Overall data showing that disrupting the microtubular network with colchicine (COLCH) does not affect cell swelling, but prevents HS-induced increase in DAF-FM fluorescence ($n = 11$ from 5 hearts). Arrows indicate superfusion change from IS to HS (except in cells maintained in IS). Results are expressed as mean \pm SEM; * $P < 0.05$ vs. cells in IS.

Di-8-ANNEPS. After processing these images with a software specifically designed to evaluate TT architecture (Auto TT, University of Iowa),¹² we found that superfusion with HS produced a small non-significant increase in TT spacing and no change in TT integrity index [transversal elements (TEs) density multiplied by TE regularity], indicating that, under our experimental conditions, cells maintain sarcolemmal and TT integrity. Although these results suggest that there is no detubulation with HS, the increase in cell volume should still promote deformation of the microtubular network, which is known to transmit mechanical cues from cell surface to TT-SR complex.¹⁶ The potential involvement of microtubules in the mechanotransduction of swelling-induced cell deformation to activation of NOS1 was assessed using myocytes preincubated for 2 h with 10 μM colchicine, a manoeuvre known to inhibit microtubule polymerization.¹⁶ Figure 3B shows overall results of cell width and DAF-FM fluorescence, indicating that inhibiting microtubule polymerization does not affect the degree of cell swelling, but prevents a HS-induced increase in NO-sensitive DAF-FM fluorescence. These results suggest that microtubule integrity is required for HS-induced NOS1 activation.

3.3 Preventing NO release exacerbates the hypotonic swelling-induced negative inotropic effect

Hypotonic swelling has been well established to produce a negative inotropic effect in adult cardiomyocytes.^{17,18} However, the underlying mechanisms are not completely understood. Given that NO has been extensively shown to modulate cardiac contractility,¹⁹ we investigated whether NO release during hypotonic swelling impacts on myocyte contractility.

Superfusing myocytes with HS produces a slow developing negative inotropic effect that reaches its maximum after ~ 15 min and then starts to recover returning to control contractility after 25 min in HS (see Supplementary material online, Figure S1). Figure 4A shows representative traces of twitch contractions and the associated Ca^{2+} transients of a cardiomyocyte stimulated to contract at 0.5 Hz under isotonic conditions and 15 min after switching to HS. Superfusing cells with HS produced a negative inotropic effect associated with a parallel decrease in the Ca^{2+} transient. When experiments were performed

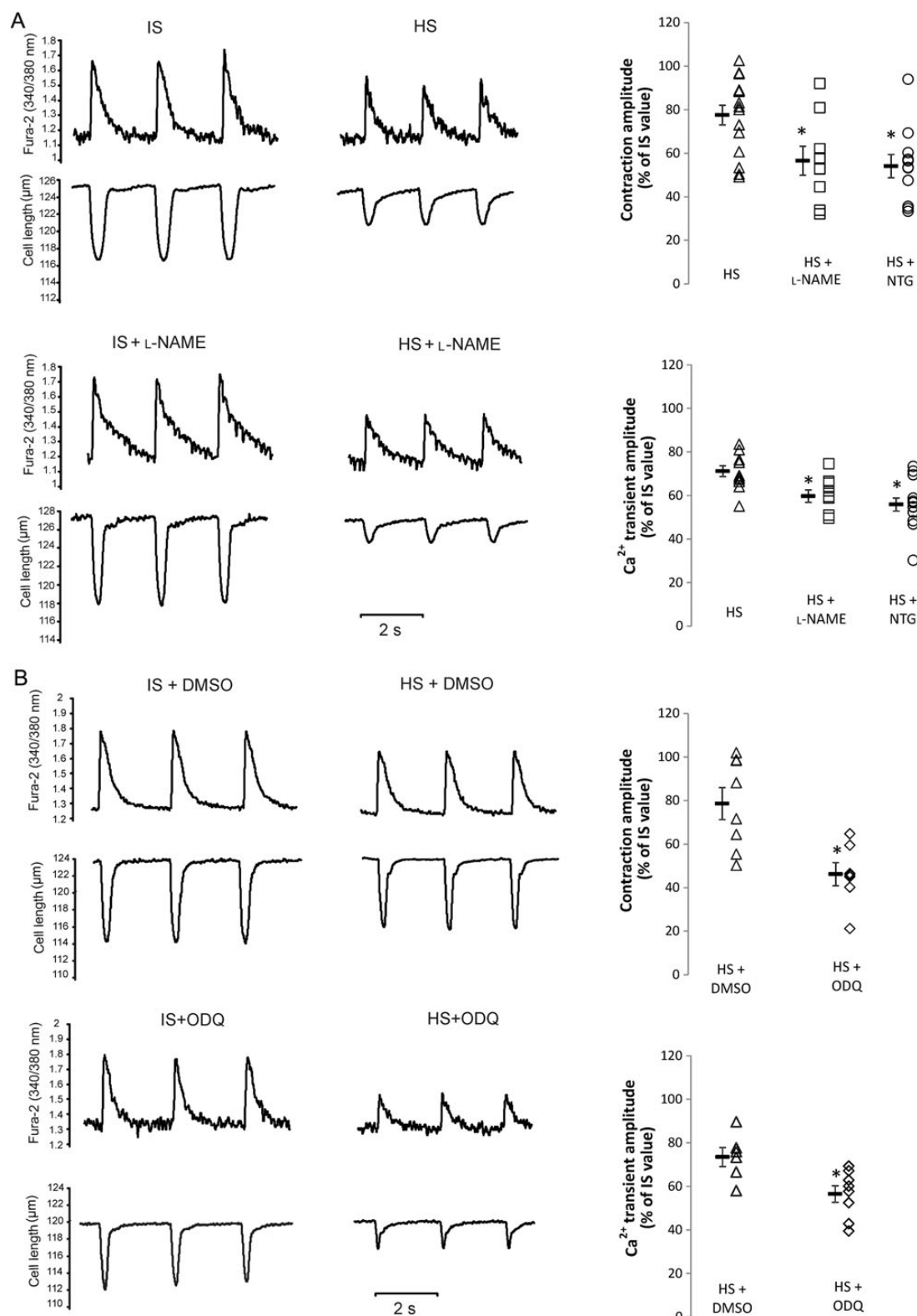


Figure 4 Inhibiting NO production exacerbates hypotonic swelling-induced negative inotropic via a cGMP-dependent mechanism. (A) Typical tracings and overall results of cell length and Ca^{2+} showing that HS reduces contraction amplitude and the associated Ca^{2+} transient ($n = 16$ from 14 hearts), and that this effect is significantly enhanced by both L-NAME ($n = 9$ from 5 hearts) and NTG ($n = 11$ from 7 hearts). Overall data at 15 min in HS, HS + L-NAME, or HS + NTG (expressed as the percent of the value in IS, IS + L-NAME, and IS + NTG, respectively). Results are expressed as mean \pm SEM; $*P < 0.05$ vs. cells in HS. (B) Typical tracings and overall results of the effect of HS on cell length and Ca^{2+} in the presence and absence of ODQ. Overall data are values at 15 min superfusion with HS ($n = 7$ from 6 hearts) or HS + ODQ ($n = 8$ from 6 hearts), expressed as the percent of the value in IS and IS + ODQ, respectively. Results are expressed as mean \pm SEM; $*P < 0.05$ vs. cells in HS. IS and HS were supplemented with DMSO to control for the vehicle used in the preparation of IS + ODQ and HS + ODQ, respectively.

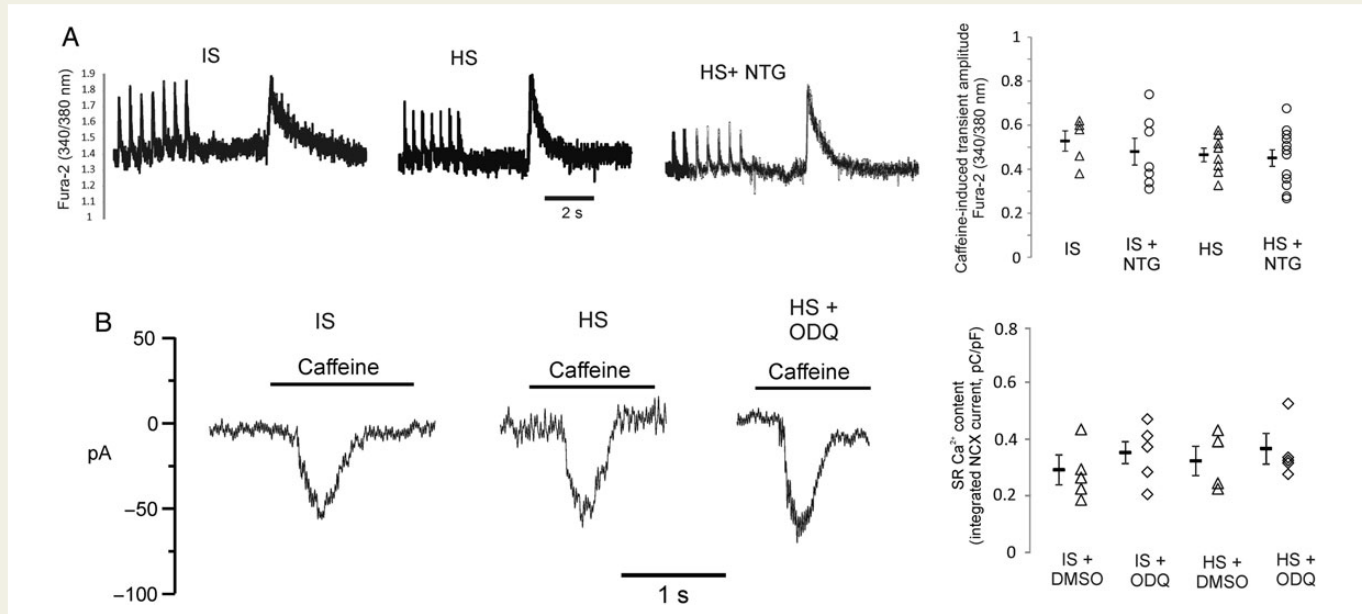


Figure 5 SR Ca^{2+} content assessed under IS and HS conditions. (A) Typical tracings and overall results of caffeine pulses performed in rat myocytes superfused for 15 min in IS ($n = 5$ from 4 hearts), IS + NTG ($n = 7$ from 4 hearts), HS ($n = 8$ from 4 hearts), or HS + NTG ($n = 12$ from 4 hearts). Neither HS nor HS + NTG significantly affected the amplitude of the caffeine-induced Ca^{2+} transient. Results are expressed as mean \pm SEM. (B) Typical traces of caffeine-induced NCX inward current under IS and HS superfusion in the presence and absence of ODQ. Overall data of the integrated NCX current normalized by cell capacitance show the failure of HS and ODQ to affect SR Ca^{2+} content. IS and HS were supplemented with DMSO to control for the vehicle used in the preparation of IS + ODQ and HS + ODQ.

in the continued presence of 2.5 mM L-NAME, the HS-induced reduction in contractility and Ca^{2+} transient were significantly exacerbated. Furthermore, the spontaneous recovery of contractility observed after 25 min of superfusion with HS was lost in cells treated with L-NAME (see Supplementary material online, Figure S1). Overall results show that both L-NAME and 240 nM NTG exacerbate the HS-induced negative inotropic effect. The addition of L-NAME or NTG did not affect contraction and Ca^{2+} transient amplitude in IS (see Supplementary material online, Figure S2). These results suggest that NOS1-dependent NO release contributes to sustain cardiomyocyte contractile function during hypotonic swelling.

3.4 cGMP mediates NO-induced contractile support

To explore the signalling cascade by which NO sustains contractile function during hypotonic swelling, we performed experiments in the presence and absence of 10 μM of the guanylate cyclase inhibitor, ODQ. ODQ did not affect contraction and Ca^{2+} transient amplitude in IS (see Supplementary material online, Figure S2) nor the degree of cell swelling (see Supplementary material online, Figure S3A). In contrast, as shown in the typical tracings and overall data depicted in Figure 4B, ODQ exacerbates the HS-induced negative inotropic effect. Interestingly, ODQ enhances the HS-induced negative inotropic effect in a similar degree than L-NAME and NTG (Figure 4A and see Supplementary material online, Figure S4), confirming not only that NOS1 is the source of NO, but also that, in the setting of hypotonic swelling, the effect of NO on myocyte contraction is mediated by the cGMP signalling and not by cGMP-independent mechanisms.

3.5 Mechanisms underlying cGMP-induced contractile support

To examine whether NO/cGMP-induced contractile support was due to alterations in SR Ca^{2+} handling, we measured SR Ca^{2+} content and RyR2 Ca^{2+} release.

Figure 5A depicts typical tracings of Fura-2 fluorescence, showing the effect of hypotonic superfusion in the absence and presence of NTG on caffeine-induced SR Ca^{2+} release. Consistent with other reports,¹⁷ superfusion with HS was associated with a small but not significant reduction in amplitude of the caffeine-induced Ca^{2+} transient, suggesting that hypotonic swelling does not largely affect SR Ca^{2+} content. Supplementary material online, Figure S3B further shows that the caffeine-induced Ca^{2+} transient was also not significantly affected by ODQ. Hypotonic swelling dilutes intracellular content, reducing cytosolic Ca^{2+} buffering. Under these conditions, a larger Fura-2 transient would be expected when Ca^{2+} is released into a less buffered compartment, masking a possible reduction in the caffeine-induced Ca^{2+} transient. To resolve this issue, we used the integral of the caffeine-induced NCX current (I_{NCX}) as a non-fluorescent readout of SR Ca^{2+} content.¹³ Figure 5B shows that superfusion with HS or HS in the presence of ODQ did not affect the caffeine-induced I_{NCX} compared with IS, confirming that neither the hypotonic swelling-induced negative inotropic effect nor contractile support provided by the NO/cGMP axis is determined by alterations in SR- Ca^{2+} content.

We next examined the impact of HS-derived NO on SR- Ca^{2+} release by monitoring Ca^{2+} spark and wave dynamics as indicators of RyR2 function. Supplementary material online, Figure S5 shows that superfusing cells with HS produces a significant decrease in Ca^{2+} spark frequency. Although not attaining statistical significance, spark frequency showed

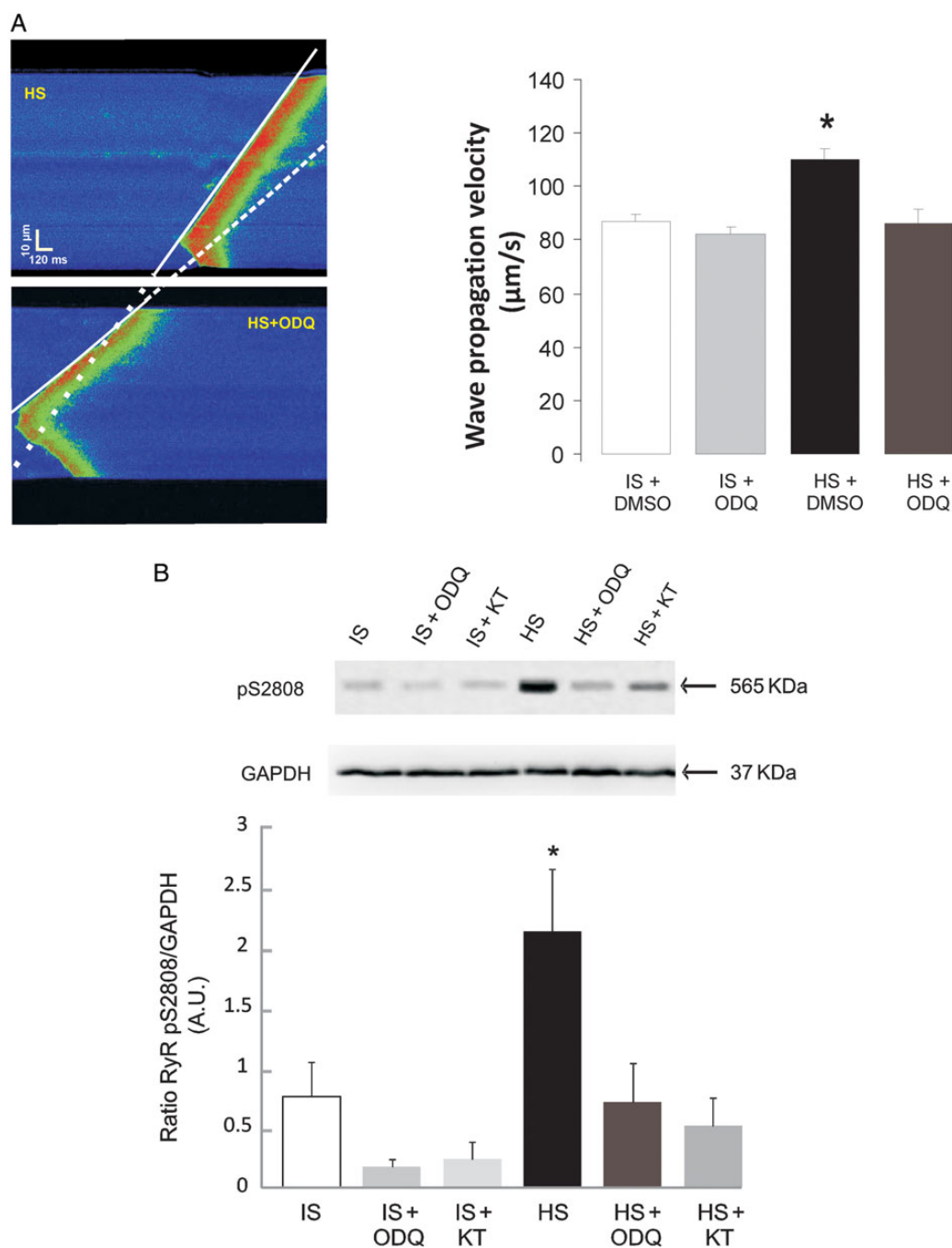


Figure 6 cGMP increases wave propagation velocity and promotes phosphorylation of RyR2 site Ser2808. (A) Typical example of a linescan image with the slopes of the Ca^{2+} wave fronts from cells in HS in the absence and presence of ODQ highlighted by a white line. The graph on the right depicts the overall results of these experiments. Values in HS represent mean data at 15 min superfusion with HS. Results are expressed as mean \pm SEM; * $P < 0.05$ vs. cells in HS alone. Control solutions were supplemented with DMSO to account for the vehicle used in ODQ solutions. (B) Representative blots and overall results showing HS increases RyR2 Ser2808 phosphorylation, and that both ODQ and KT5823 prevent this increase. Data are expressed as means \pm SEM from seven independent experiments from six hearts. * $P < 0.05$ vs. all groups.

a tendency to be further reduced in the presence of ODQ. The failure to observe a significant effect of ODQ on Ca^{2+} spark frequency could be due to the fact that as swelling *per se* decreases spark frequency, a further decrease in the presence of ODQ could be difficult to detect. Thus, we evaluated the velocity of Ca^{2+} wave propagation in cardiomyocytes as a

measure of RyR2 opening properties in response to cytosolic Ca^{2+} .²⁰ Figure 6A depicts typical linescan images of Ca^{2+} wave propagation in myocytes superfused for 20 min in HS either in the absence or presence of 10 μM ODQ, showing that ODQ reduces Ca^{2+} wave velocity in HS. Overall results show that whereas ODQ does not affect wave velocity

in IS, HS produces a significant increase in wave velocity, which is prevented by ODQ. These results suggest that NO could be sensitizing RyR2 to cytosolic Ca^{2+} during hypotonic swelling.

To determine how cGMP may be affecting RyR2 function, we examined the effect of HS on the phosphorylation status of the PKA/PKG-dependent RyR2 residue, Ser2808. Interestingly, Figure 6B shows that HS significantly increases Ser2808 phosphorylation, and that this increase is prevented by both 10 μM ODQ and 1 μM of the PKG inhibitor, KT5823. To examine whether PKG-dependent phosphorylations are involved in the inotropic support induced by NO, we evaluated the effect of HS on contraction amplitude in the presence of KT5823. Supplementary material online, Figure S6 shows that similar to L-NAME, NTG, and ODQ, inhibiting PKG exacerbates the hypotonic swelling-induced negative inotropic effect.

4. Discussion

The present study demonstrates, for the first time, that cell swelling promotes NO release in ventricular cardiomyocytes. Our results also suggest that NOS1 would be the primary NOS isoform responsible for NO release, being microtubule integrity required for the mechanotransduction of swelling-induced NO release. Furthermore, our results suggest that NO plays a functional role by reducing the hypotonic swelling-induced negative inotropic effect. We propose that NO via cGMP/PKG signalling enhances ryanodine receptor sensitivity to trigger Ca^{2+} , which could contribute to sustain contractile function during hypotonic swelling.

4.1 Hypotonic swelling promotes NO production in rat ventricular cardiomyocytes

Recent reports in tissues other than cardiac have shown evidence, indicating that cell swelling can promote NO release.^{7,8} Moreover, we have previously reported that cardiomyocyte axial stretch promotes NO release.⁶ However, cardiomyocyte swelling dilutes intracellular contents and deforms the surface membrane without significant changes in cell length. Thus, extrapolating our results from a myocyte stretch model to one of cell swelling is not straightforward.

Here, we show that superfusion of cardiomyocytes with a HS, similar to other reports,¹⁰ increases cell width, indicative of hypotonic cell swelling. This cellular swelling is associated with an increase in the fluorescence of the NO sensor, DAF-FM, which was not observed when cells were superfused with IS. In addition, whereas NOS inhibition prevented the HS-induced increase in DAF-FM fluorescence, it did not prevent cell swelling, confirming that NO production is due to the activation of NOS.

There are three known isoforms of NOS within the myocardium, NOS1, NOS2, and NOS3; of which NOS1 and NOS3 are constitutively expressed.⁵ Inducible NOS or NOS2 is mainly expressed during inflammatory processes.²¹ To determine which NOS isoform is involved in hypotonic swelling-induced NO release, we used (i) the NOS1-specific inhibitor, NTG and (ii) the PI3K inhibitor, Wortmannin, to inhibit NOS3. This inhibitor was used taking into consideration that cardiomyocyte swelling is associated with a reduction in the Ca^{2+} transient,^{17,18} and that recent reports indicate that activation of the PI3/AKT axis can alter the Ca^{2+} sensitivity of NOS3 enabling NO production without a significant rise in $[\text{Ca}^{2+}]_i$.²² Both inhibitors failed to significantly affect the degree of cell swelling. However, whereas Wortmannin did not

significantly affect HS-induced NO release, NTG completely prevented the increase in DAF-FM fluorescence. It is important to note that the fact that none of the inhibitors used, affected the degree of cell swelling, excludes the possibility that the failure to observe an increase in DAF-FM fluorescence was due to a reduction in cell swelling. Taken together, these results suggest that NOS1 is the main enzyme involved in hypotonic swelling-induced NO release. Nevertheless, these findings are at odds with a recent report indicating that, in endothelial cells, hypotonic swelling-induced NO production is mediated by Ca^{2+} -independent activation of NOS3.⁷ To validate our observation, we used two additional approaches: (i) given that NOS1 activation requires a functional SR, we treated myocytes with the RyR2 inhibitor, ryanodine (Ry), plus the SR Ca^{2+} pump (SERCA2A) inhibitor, CPA, an intervention known to abolish SR function. (ii) We performed experiments in myocytes from NOS1 knockout mice and their WT controls. Confirming the critical role played by NOS1, we found that HS-induced NO production was prevented in the presence of Ry + CPA and significantly reduced in myocytes from NOS1 knockout mice (Figure 2C and D). Interestingly, the failure to observe an increase in NO production in myocytes treated with Ry + CPA further suggests that the NOS1 isoform involved is the one localized at the SR and not the recently described mitochondrial NOS1.¹⁵ Further insights into the mechanism underlying NOS1 activation are provided by the results showing that the inhibition of microtubule polymerization was able to prevent NO release, suggesting that microtubule integrity is required for hypotonic swelling NOS1 activation (Figure 3B). Although the mechanism by which the cytoskeleton may promote NOS1 activation is not clear, given the close proximity of microtubules with the SR (10^{-8} m)¹⁶ it could be proposed that microtubules could intervene in the physical transmission of stress or strain from the sarcolemma to the SR resulting in NOS1 activation. Supporting this contention, various components of the cytoskeleton network have been shown to modulate NOS activity and NO production.²³

4.2 Impact of hypotonic swelling-induced NO release on contractile function

Osmotic swelling has been shown to reduce cardiomyocyte contractility, attributed mainly to a reduction in the Ca^{2+} transient.^{17,18} Brette et al.¹⁷ showed that the slow reduction in the Ca^{2+} transient induced by HS was mediated by a reduction in L-type calcium current and a decrease in SR Ca^{2+} release in the absence of changes in SR Ca^{2+} content. Consistently, our results show that HS produces a slow developing reduction in contractility associated with a reduction in the Ca^{2+} transient (Figure 4). Interestingly we also show that, after reaching its maximal negative inotropic effect, contractility and Ca^{2+} transient, in the continued presence of HS, start to recover in spite of the lack of RVD (see Supplementary material online, Figure S1).

Given that NO has been shown to have varied effects on cardiac ECC, determined at least in part by different subcellular compartmentalization of NOS isoforms,²⁴ we examined whether hypotonic swelling-induced NO release had a functional impact on the negative inotropic effect observed during this process. Here, we show that inhibiting NO production enhances the HS-induced negative inotropic effect and prevents the contractile recovery observed when cells are maintained in HS for extended periods of time (see Supplementary material online, Figure S2). Although our results do not shed mechanistic insights into the mechanism that determine the hypotonic swelling-induced negative inotropic effect, the results presented here suggest that NOS1-derived NO

provides a mechanism of contractile support that prevents an even greater impact of cell swelling on cardiac contractile function.

It is now well established that NO may modulate cardiac ECC both through cGMP-dependent and cGMP-independent mechanisms.²⁵ Our results, showing that guanylate cyclase inhibition mimics the effect of inhibiting NOS, demonstrates that cGMP-dependent mechanisms mediate NO-dependent contractile support and contractile recovery observed during hypotonic swelling.

Consistent with other reports,¹⁷ under our experimental conditions, hypotonic swelling-induced negative inotropic effect was associated with a small, not significant, reduction in the caffeine-induced Ca^{2+} transient (Figure 4B). However, given the non-linear relationship between ratio and calcium, this does not necessarily mean that SR Ca^{2+} content was unaltered. Moreover, due to a possible swelling-induced reduction in Ca^{2+} buffering, which would alter free cytosolic Ca^{2+} levels and thus Fura-2 fluorescence ratio, the possibility of a reduction in SR Ca^{2+} content contributing to the hypotonic swelling-induced negative inotropic effect cannot be excluded by this approach. Thus, to more precisely address SR Ca^{2+} content, we used the integral of the caffeine-induced NCX current as a reliable index of SR Ca^{2+} load.¹³ These experiments confirmed that hypotonic swelling was not associated with a significant reduction in SR Ca^{2+} content.

To verify whether, NO-induced contractile support was due, at least in part, to a cGMP-dependent stimulatory effect on RyR2 Ca^{2+} release, we monitored Ca^{2+} spark dynamics under IS and HS conditions in the absence and presence of ODQ. Although we made the interesting, and until now undescribed, observation that hypotonic swelling, in itself, reduces Ca^{2+} spark frequency, we were unable to detect a significant further decrease in Ca^{2+} spark frequency in the presence of ODQ (see Supplementary material online, Figure S3). However, we found that, during hypotonic swelling, when Ca^{2+} waves appear, their propagation velocity is significantly enhanced compared with the isotonic condition and that this increase is prevented by ODQ (Figure 6A). Ca^{2+} waves represent SR Ca^{2+} release triggered by cytosolic Ca^{2+} released from neighbouring RyR2 clusters. Therefore, the velocity of Ca^{2+} waves can be used as an index of RyR2 opening properties during Ca^{2+} release triggered by cytosolic Ca^{2+} .²¹ Thus, these results suggest that NO through a cGMP-dependent mechanism could be sensitizing the RyR2, favouring SR Ca^{2+} release for a given Ca^{2+} trigger. Given that ODQ did not affect SR Ca^{2+} content (see Supplementary material online, Figure S3), it seems unlikely that the observed reduction in Ca^{2+} wave velocity is determined by an ODQ-dependent reduction in SR Ca^{2+} load. The possibility that NO/cGMP acts at the level of the Ca^{2+} trigger for Ca^{2+} release, namely the L-type Ca^{2+} current, also seems unlikely based on a recent report, showing that hypotonic swelling does not affect this current via a cGMP-dependent mechanism.²⁶ Thus, in the context of cell swelling, an increase in RyR2 sensitivity could help to sustain SR Ca^{2+} release in spite of the well-described reduction in L-type Ca^{2+} current^{17,18,26} and hence contribute to sustain the Ca^{2+} transient and contractile function. It is noteworthy that an increase in RyR2 open probability as the only mechanism responsible for a positive inotropic effect is presently under debate.²⁷ Although our results suggest that RyR2 sensitization could have a causal role in the inotropic support provided by NO, we cannot exclude that NO could also have additional targets that could contribute to sustain contractile function during hypotonic swelling.

Phosphorylation of RyR2 at site Ser2808 has been shown to increase the velocity of wave propagation.²⁸ Interestingly, this site is classically considered to be phosphorylated by PKA. However, *in vitro* experiments suggest that Ser2808 can also be phosphorylated by PKG.²⁹ Our results

show that RyR2 Ser2808 phosphorylation is increased by hypotonic swelling and that this increase is prevented by inhibiting cGMP (Figure 6B), suggesting that NO/cGMP-dependent phosphorylation of this site could be responsible for sensitizing the RyR2 to cytosolic Ca^{2+} during hypotonic swelling. cGMP-dependent phosphorylation of Ser 2808 could be mediated by direct activation of PKG or by PKA, given that cGMP has also been shown to inhibit phosphodiesterases (PDEs) increasing cAMP availability and thus activating PKA.³⁰ Importantly, our results using the specific PKG inhibitor, KT5823, show that PKG phosphorylates RyR2 Ser2808, demonstrating, for the first time, the PKG-dependent phosphorylation of this site in a cardiac cellular model. Our results showing that KT5823, similar to L-NAME, NTG, and ODQ exacerbate the HS-induced negative inotropic effect (see Supplementary material online, Figure S4) indicate that NO provides contractile support, in the context of hypotonic swelling, via a cGMP/PKG-dependent mechanism. Interestingly, the late contractile recovery observed with prolonged HS superfusion was also prevented by KT5823 treatment, suggesting that this recovery is also mediated by cGMP/PKG-dependent mechanisms. However, we cannot exclude that other PKG-dependent mechanisms aside from the one described here (enhancement of RyR2 Ca^{2+} sensitivity) could be involved in this recovery.

In summary, our results show for the first time that hypotonic swelling promotes NOS1-dependent NO release in cardiomyocytes. This NO could have multiple consequences regulating distinct cellular functions through both cGMP-dependent and independent mechanisms. Highlighting the physiopathological relevance of the NO release we described herein, we show its impact on swelling-induced contractile dysfunction, where it provides inotropic support by a cGMP/PKG-dependent mechanism. This mechanism may help to sustain contractile function in different pathological situations associated with cell swelling.

4.3 Limitations

In this study, we provide evidence showing that hypotonic swelling promotes NOS1-dependent NO release. Our results using colchicine to disrupt microtubules further suggest that the microtubular network may be involved in the mechanotransduction of cell swelling to NOS1 activation. However, the observation of a lag between cell swelling and NO production could suggest that other mechanisms, in addition to mechanical forces, could be involved. Nevertheless, this interpretation of a more complex scenario could arise from a limitation in NO detection. Indeed, the observed lag could be due a lower sensitivity for NO detection compared with that for cell width detection. Thus, it could be conceivable that NO production could have a rather similar time course to that of changes in cell width, but is not detected until it builds up inside the cell reaching the threshold for detection. Clearly, these issues indicate that exploring the mechanisms involved in the mechanotransduction of NO release during hypotonic swelling deserves further study.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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