Original Paper

Role of Calcium Channels in the Protective Effect of Hydrogen Sulfide in Rat Cardiomyoblasts

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Key Words

Hydrogen sulfide • Oxidative stress • Voltage-operated calcium channels • Cardiomyoblasts • Calcium signaling

Abstract

Background: Hydrogen sulfide contributes to the reduction of oxidative stress-related injury in cardiomyocytes but the underlying mechanism is still unclear. ***Aims:*** Here we investigated the role of voltage-operated calcium channels (VOCCs) as mediators of the beneficial effect of H_2S against oxidative stress in cultured rat cardiomyoblasts (H9c2). ***Methods:*** Intracellular calcium signals were measured by fluorimetric live cell imaging and cell viability by colorimetric assay. ***Results:*** Treatment with H_2S donor (NaHS 10 µM) or Nifedipine (10 µM) decreased resting intracellular calcium concentration $[Ca]_i$, suggesting that L-type VOCCs are negatively modulated by H_2S. In the presence of Nifedipine H_2S was still able to lower $[Ca]_i$, while co-incubation with Nifedipine and Ni^{2+} 100 µM completely prevented H_2S-dependent $[Ca]_i$ decrease, suggesting that both L-type and T-type VOCCs are inhibited by H_2S. In addition, in the same experimental conditions, H_2S triggered a slow increase of $[Ca]_i$ whose molecular nature remains to be clarified. Pretreatment of H9c2 with NaHS (10 µM) significantly prevented cell death induced by H_2O_2. This effect was mimicked by pretreatment with L-Type calcium channel inhibitor Nifedipine (10 µM). ***Conclusions:*** The data provide the first evidence that H_2S protects rat cardiomyoblasts against oxidative challenge through the inhibition of L-type calcium channels.

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Introduction

Oxidative stress is the principal cause of cell death during ischemia/reperfusion (I/R) processes [1-5]. In fact, restoration of blood flow and oxygen supply generates reactive oxygen species (ROS) that trigger apoptosis and necrosis phenomena [6-8]. Cardiomyocytes face this occurrence during the so-called myocardial infarction consisting of I/R in the cardiac tissue. ROS form during re-oxygenation of the infarcted tissue and lead to the activation of a wide range of reaction cascades resulting in pro-apoptotic and necrotic stimuli [9, 10]. Membranes integrity is compromised by phospholipids oxidation leading to uncontrolled ionic permeability [8]. Ionic conductance can also be affected by the direct action of ROS on channel proteins [11]. The effect of such processes is, among the others, an increase Ca^{2+} permeability with a consequent rise of intracellular calcium levels. It is not clear, however, whether voltage-operated calcium channels (VOCC) plays a role in myocardial I/R injury [12]. Consistently, in vitro experimental models confirmed the efficacy of H_2S donors in protecting cultured cardiomyocytes against hypoxia/reoxygenation and oxidative challenges [13]. Several mechanisms have been proposed to explain such outcome but literature on this topic is still controversial and laconical [14].

On the other hand, what appears unequivocal is that H_2S can regulate the activity of ion channels either in the outer membrane or in intracellular organelles such as mitochondria [15]. The first report on the effect of H_2S on ion channels originally appeared more than one decade ago [16] and, since then, the modulation of plasmalemma calcium channels by H_2S has been shown [17]. In cardiomyocytes, two predominant calcium channels are expressed: L-type and T-Type. L-type Ca^{2+} channels are ubiquitously expressed representing the principal mediator for excitation–contraction coupling in the heart. Concurrently, T-type Ca^{2+} channels are selectively expressed depending on species, cardiac region and pathological states. In I/R injury, for instance, calcium channels activity is altered to mediate post-ischemic deleterious effects [18]. In fact, one of the most detrimental factors of interrupting and restoring blood supply to the heart is the unbalanced change in calcium homeostasis [19]. Calcium overload in post-ischemic cardiomyocytes is the leading cause of cell death either through apoptosis or necrosis [20]. Intracellular calcium concentration is finely regulated through the control of flux from the extracellular compartment and from internal reservoirs. Alteration of this subtle balance triggers a plethora of effects and H_2S can, indeed, cause physiological changes of calcium permeability. In our previous reports we showed that the administration of NaHS induces a significant increase in intracellular calcium in endothelial cells from excised rat aorta [21]. On the other hand, in pancreatic beta cells NaHS inhibited L-type VOCCs [22].

In the present study we investigate the role of H_2S in the regulation of VOCCs and the related functional effects on the cardiomyoblast cell line H9c2.

Materials and Methods

Cell cultures

Rat embryonic ventricular myocytes cells line H9c2 were obtained from American Collection of Cell Cultures (ATCC® CRL-1446™ Milan Italy). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 µg/mL gentamycin and 4 mM l-glutamine, and maintained in normal culture conditions (37°C, saturated humidity atmosphere at 95% air/5% CO_2). Before reaching confluence, cells were sub-cultured onto 25 cm² culture flasks and used at passages 2–7.

H2S donor and Stressor compound

Sodium Hydrogen Sulfide (NaHS) and Hydrogen peroxide (H_2O_2) were purchased from Sigma Aldrich and freshly prepared on the day of the experiments by dissolving the salt in the physiological solution (TS) for calcium imaging experiments and/or in the culturing medium for MTT assay.

Cell viability assay

The cell viability was analyzed using a methylthiazol tetrazolium (MTT) assay. The colorimetric assay is based on the ability of live cells to reduce the yellow MTT reagent (Sigma Aldrich, Italy) to a purple formazan product. H9c2s were seeded in 96-well plates at a cellular density of $0.5x10^4$ cell/well. Cells were starved overnight with 1% FCS in order to increase cell cycle synchronization. The following day H9c2 were treated for 1 hour with different concentrations of NaHS and/or 10 μM Nifedipine alone. Afterward, cells were exposed to 50 μM H_2O_2 (3 hour) in order to induce oxidative stress. A total of 100 μl of MTT solution was added to each well, and the cells were then incubated at 37°C, 5% CO_2 for 4 h. After incubation, MTT was aspirated and 100μl/well DMSO was added. Subsequently, the cell viability was assessed by measuring the absorbance at 570 nm.

Calcium imaging

For ratiometric $[Ca]_i$ measurements on H9c2, cells were loaded with Fura-2/AM as previously described [1]. $[Ca]_i$ was expressed as a ratio (R) of emitted fluorescence corresponding to excitation wavelengths of 340 nm and 380 nm.

High-potassium solution (HK) was prepared as follows: NaCl (118 mM), KCl (40 mM), $CaCl_2$ (2 mM), $MgCl_2$ (1 mM), glucose (5.5 mM), Hepes (5 mM), and NaOH to pH 7.35.

During the experiments, H9c2 were maintained in standard Tyrode solution (TS) of the following composition (in mmol/L): NaCl, 154; KCl, 4; $CaCl_2$, 2; $MgCl_2$, 1; HEPES, 5; glucose, 5.5; and NaOH (pH 7.35); for experiments in calcium-free conditions, the external solution was modified omitting the $CaCl_2$ salt from the formulation and adding the Ca^{2+} chelator EGTA (5 mmol/L).

The experiments were performed at room temperature. Amplitude of resting $[Ca]_i$ decrease was evaluated as the difference between $[Ca]_i$ before treatment and the minimum R value measured during treatment. $[Ca]_i$ increase was evaluated as the difference between the maximal $[Ca]_i$ value reached during the treatment (peak) and resting $[Ca]_i$ before treatment. In calcium imaging experiments with H_2O_2, the slope of $[Ca]_i$ increase during the treatment was evaluated by the use of IgorPro software (Wavemetrics, USA). Any alteration of Fura-2/AM functionality due to H_2O_2 was excluded by separately analyzing the emission corresponding to 340 nm and 380 nm excitation.

Data analysis and statistics

For Fura-2 ratiometric measurements, single cells were selected for each image sequence with Metafluor software (Universal Imaging Corporation).

Calcium imaging offline analyses were performed using IgorPro software. Statistical significance of all experiments was evaluated with Kaleidagraph software (Synergy Software, USA). We used nonparametric tests (Wilcoxon) for the entire statistical analysis in this work since not all the data were normally distributed.

Results

H2S inhibits L-type calcium channels in H9c2

Acute application of NaHS (1-100 μM) to H9c2 cells bathed in Tyrode Standard (TS) physiological extracellular solution induced a large and reversible decrease of resting intracellular calcium ($[Ca]_i$) in 60% of tested cells ("NaHS-sensitive cells"): Figure 1A shows a representative response to 10 μM NaHS. Interestingly, NaHS-sensitive cells had a higher resting $[Ca]_i$ compared to insensitive cells (R=0.52 *vs*. 0.48, as shown in Figure 1B, grey bars).

When cells were exposed to high-potassium (HK) extracellular solution (40 mM KCl, see methods) in order to depolarize cell membrane and to activate all the VOCCs, the effect of NaHS was detected in a significantly higher subpopulation of cells (≥80%). In HK solution, as in the case of TS-bathed cells, NaHS-sensitive population displays higher resting $[Ca]_i$ than insensitive cells (R=0.56 vs. 0.49, Fig. 1B, black bars).

In particular, resting $[Ca]_i$ decrease triggered by 1, 10 and 100 μM NaHS was observed respectively in 68.7%, 78,0% and 78,5% of the cells.
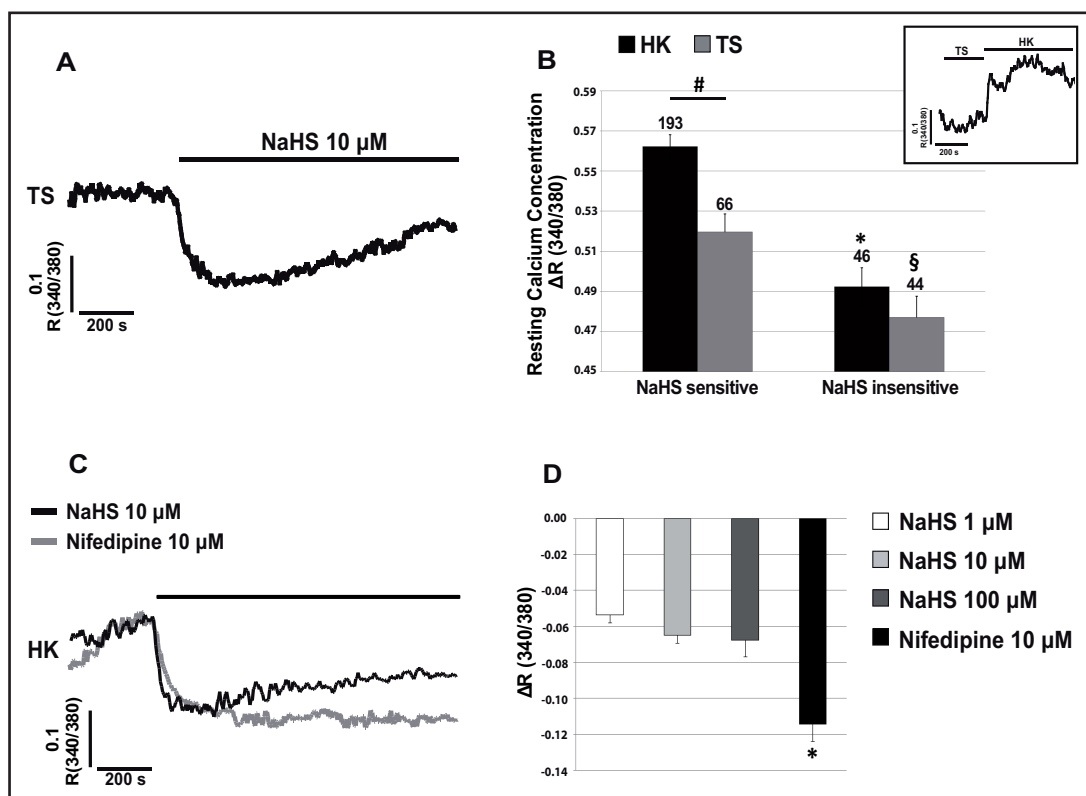
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Fig. 1. Effect of NaHS on resting $[Ca]_i$ in H9c2. Representative trace showing the decrease in resting $[Ca]_i$ induced by acute application of 10 μM NaHS in cells bathed in a physiological extracellular solution (TS) (A). Resting $[Ca]_i$ levels in cells sensitive or not to NaHS treatment (NaHS-sensitive and NaHS-insensitive) bathed in high potassium solution (HK, black bars) or in physiological extracellular solution (TS, grey bars). Cell numbers are reported on the top of histogram. Wilcoxon test. HK versus TS in NaHS-sensitive cells #p<0.01 (a representative trace of the acute effect of HK perfusion is shown in the inset of figure 1B). NaHS-sensitive versus NaHS-insensitive cells in HK solution *p<0.001. NaHS-sensitive versus NaHS-insensitive in TS solution §p<0.001 (B). Representative traces showing the decrease in resting $[Ca]_i$ induced by acute application of 10 μM NaHS (black line) or 10 μM Nifedipine (grey line) in cells bathed in HK solution (C). Quantification of $[Ca]_i$ decrease induced in HK-bathed cells by three NaHS concentrations (1-10-100 μM) or 10μM Nifedipine (D). Wilcoxon test. NaHS (1-10-100 μM) versus Nifedipine 10 μM *p<0.001. Data are expressed as the mean ± S.E.M.

Acute perfusion with 10 μM Nifedipine, a selective blocker of L-type calcium channels, induced a resting $[Ca]_i$ decrease in 100% of H9c2s bathed in HK solution: the amplitude of the effect was higher than that observed upon NaHS treatment (Fig. 1C and 1 D).

Resting $[Ca]_i$ of NaHS-sensitive cells pretreated with Nifedipine was significantly lower than that measured in cells untreated with the blocker. On the other hand, no significant differences were detected between NaHS-insensitive cells treated or untreated with Nifedipine (Fig. 2A) in agreement with an earlier report [23]. In particular, our data suggest that H₂S-sensitive L-type VOCCs are active in resting conditions in a subpopulation of H9c2 cells.

H2S inhibits T-type Calcium Channels

Interestingly, in cells pre-incubated with 10 μM Nifedipine, acute application of 10 μM NaHS was still able to induce a slow, although significant, reduction of resting $[Ca]_i$ in a percentage lower than that observed in Nifedipine-untreated cells (Fig. 2B and Fig. 3A). Since T-type VOCC are expressed in cardiomyocytes [24], we decided to investigate their putative role as a target for NaHS in H9c2 cells under resting conditions.

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Fig. 2. Effect of inhibition of L-type calcium channels. Histogram reporting the resting $[Ca]_i$ levels in NaHS-sensitive and NaHS-insensitive HK-bathed cells preincubated or not with 10 μM Nifedipine. (A). NaHS-induced $[Ca]_i$ decrease in HK-bathed cells pre-incubated with 10 μM Nifedipine (white bar) or not (black bar) (B). Data are expressed as the mean ± S.E.M. Wilcoxon test *$p<0.001$.
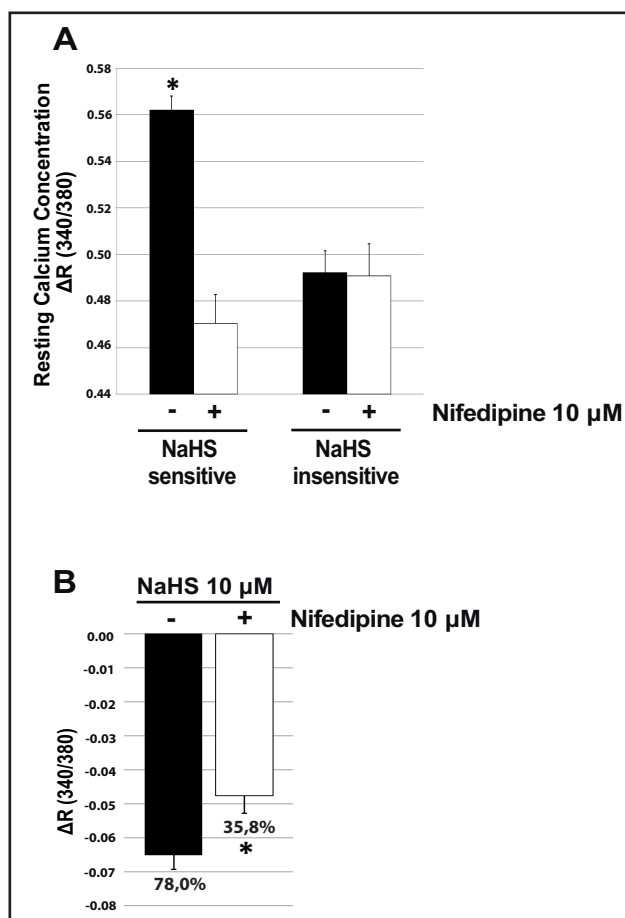
In two distinct sets of experiments we used two different Ni^{2+} concentrations, 30 μM and 100 μM, respectively acting on only subtype α1H or on the totality of T-type channels (α1H, G and I) [25].

In cells pretreated with Nifedipine and 30 μM Ni^{2+} acute application of 10 μM NaHS was still able to decrease resting $[Ca]_i$ in 90% of the cells, but the effect was lower than that observed in cells pre-treated with Nifedipine only (Fig. 3A).

On the other hand, in cells pretreated with Nifedipine and 100 μM Ni^{2+}, acute application of 10 μM NaHS completely failed to decrease resting $[Ca]_i$ levels (Fig. 3A). Accordingly, cells pretreated with both 10 μM Nifedipine and 100 Ni^{2+} showed resting $[Ca]_i$ levels significantly lower than untreated cells. Switching to free calcium extracellular medium did not affect resting $[Ca]_i$ (Fig. 3B). Furthermore, acute application of 10 μM NaHS induced a $[Ca]_i$ increase in 100% of cells pretreated with both Nifedipine and 100 μM Ni^{2+}. The amplitude of the response was higher in TS solution then in HK solution and resulted drastically reduced in free-calcium extracellular medium (Fig. 3C, 3D).

H2S prevents H9c2 death induced by H2O2 via L-type calcium channels

H9c2 cells were challenged for 3 hours with 50 μM H_2O_2 in order to promote oxidative stress-induced cell death (59% cell survival). Pre-incubation for 1 hour with different concentrations of NaHS (1, 10, 100 μM) enhanced cell survival of 55±3%, 58±2% and 78±3% respectively (black bar in Fig. 4). Similar results were obtained when NaHS was used as a post-conditioning agent, in which cells were challenged with H_2O_2 (50 μM, 1 hour) and then treated with NaHS (data not shown).

All the concentrations tested were able to protect the cells against H_2O_2 induced-apoptosis (Fig. 4, left panel). H_2S *per se* did not significantly affect cell viability (not shown).

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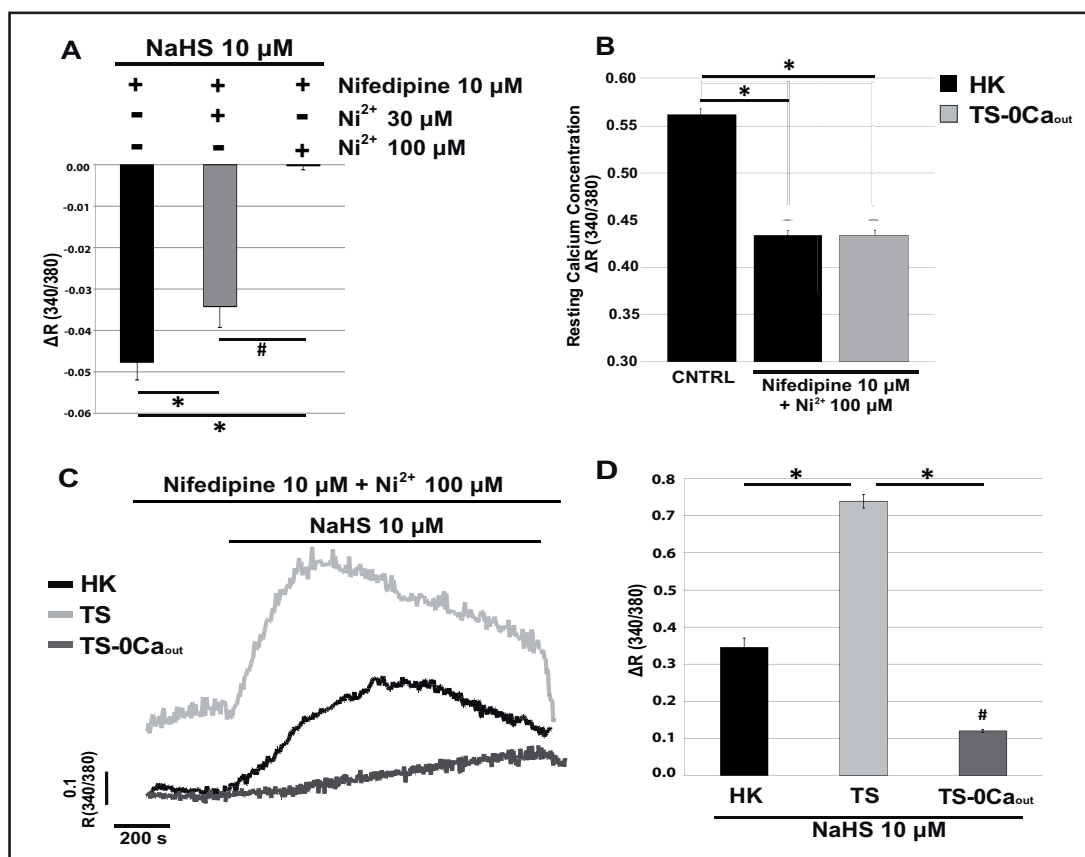

Fig. 3. Effect of inhibition of L- and T-type calcium channels. Histogram reporting the amplitude of NaHS-induced $[Ca]_i$ decrease in cells pre-incubated with 10 µM Nifedipine and 30 µM Ni^{2+} or 100 µM Ni^{2+} (A). Resting $[Ca]_i$ levels in cells preincubated with 100 µM Nickel and 10 µM Nifedipine and bathed in HK or free calcium-TS solution. CNTRL is resting $[Ca]_i$ in not-preincubated cells (B). Representative traces showing NaHS-induced $[Ca]_i$ increase in cells pre-incubated with 10 µM Nifedipine and 100 µM Ni^{2+}; black trace: HK-bathed cells, light gray trace: TS-bathed cells and dark grey: TS-calciumf-ree-bathed cells (C). $[Ca]_i$ increase has been quantified measuring the peak amplitude for each condition (D). Wilcoxon test. *$p<0.001$. Data are expressed as the mean ± S.E.M.

Fig. 4. Effect of NaHS on cell viability upon oxidative stress. Left panel. Cell survival after 1 hour pre-incubation different NaHS concentrations followed by oxidative challenge with 50µM H_2O_2. Right panel. Cell survival after 1 hour co-incubation with different NaHS concentrations and/or 10 µM Nifedipine alone followed by oxidative challenge with 50µM H_2O_2. Wilcoxon test. CTRL (black box) is significantly different compared to all other conditions tested (**, $p<0,001$). N=3 Data are expressed as the mean ± S.E.M.
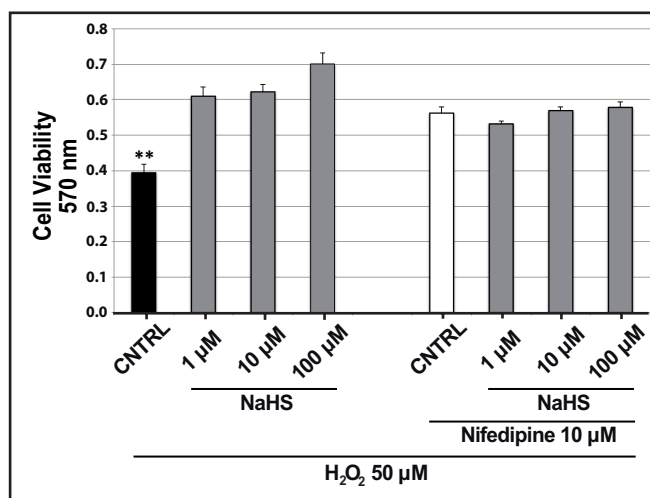
Similarly to NaHS, pre-incubation with 10 µM Nifedipine protected cells from H_2O_2 challenge (white bar in Fig. 4). Interestingly, no additive effect could be observed when cells were
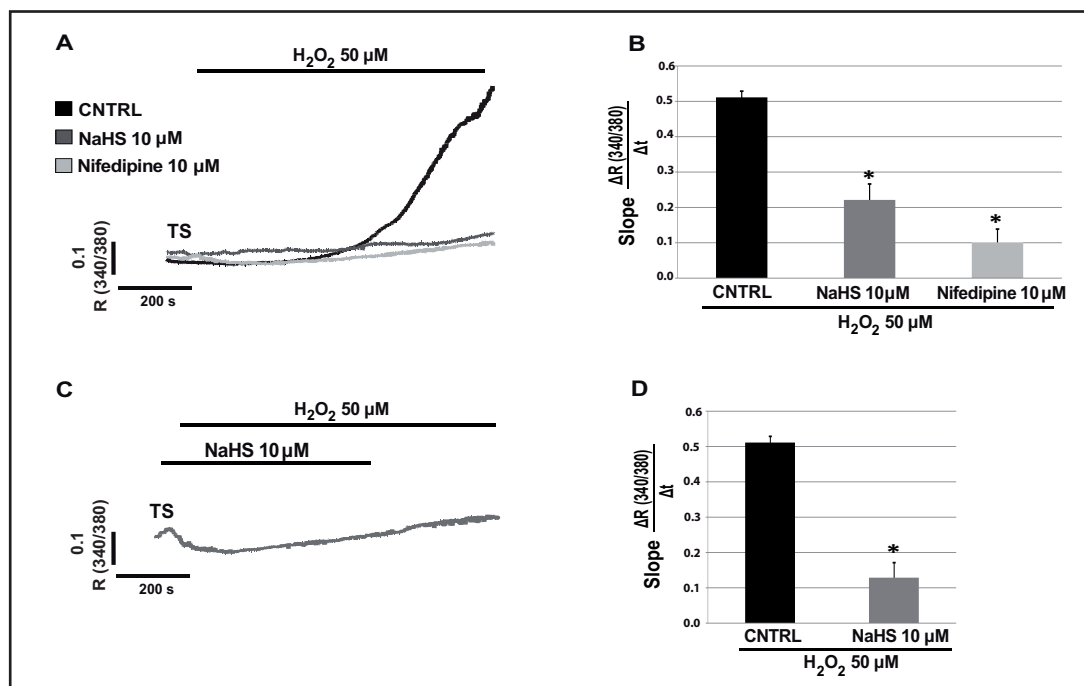
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Fig. 5. Effects of H_2O_2 on $[Ca]_i$. Representative traces showing the effect of 50 µM H_2O_2 perfusion on $[Ca]_i$ in control cells (black trace), 10 µM NaHS-preincubated cells (dark grey) and 10 µM Nifedipine-preincubated cells (light grey) (A). Histogram showing a quantification of the slope in $[Ca]_i$ increase. *$p<0.001$ versus CNTRL. (B). Representative trace and quantification of $[Ca]_i$ increase induced by 50 µM H_2O_2 in cells in which NaHS was perfused starting from 10 minutes after oxidative challenge (C and D). Wilcoxon test. *$p<0.001$ versus CNTRL. Data are expressed as the mean ± S.E.M.
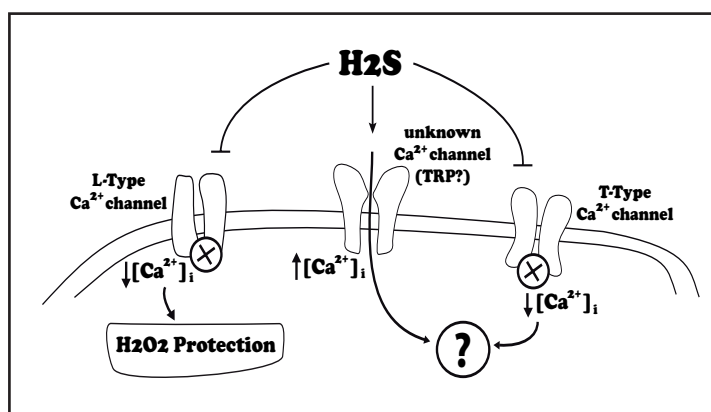

Fig.6. Scheme reviewing the main conclusions of the work.

co-incubated with 10 µM Nifedipine and NaHS (1 µM, 10 µM and 100 µM), suggesting a convergence between L-type calcium channels and H_2S-mediated effects (Fig. 4, right panel).

We performed calcium imaging experiments in order to evaluate $[Ca]_i$ changes during oxidative stress (Fig. 5). Acute perfusion with 50 µM H_2O_2 induced a delayed and irreversible $[Ca]_i$ increase, detectable starting from 30-40 minutes of H_2O_2 perfusion. Pre-incubation with either 10 µM NaHS or 10 µM Nifedipine largely prevented H_2O_2-induced $[Ca]_i$ increase (Fig. 5A and 5B). The same protective effect against the large $[Ca]_i$ increase was observed when 10 µM NaHS was perfused starting from 10 minutes after oxidative challenge (Fig. 5 C and D).

Discussion

Reoxygenation-driven oxidative stress is responsible for post-ischemic cell death. Restoration of blood supply after ischemia triggers massive tissue damage leading to impaired organ function. Myocardial and cerebral ischemia are estimated to be the two major causes of death in the western countries with a profound socio-economic burden. Therefore, the comprehension of mechanisms underlying this process and the investigation of possible treatments appears to be of paramount importance.

Hydrogen sulfide is an important gasotransmitter [26]. Although several reports confirm the efficacy of H$_2$S in reducing reoxygenation and reperfusion injury in the myocardium and a large body of literature has been dedicated to putative mechanisms [27], we still lack pharmacological targets that can translate these basic research findings into clinical and therapeutic applications. We selected an immortalized cardiomyoblast cell line (H9c2) that represents one of the few valid *in vitro* models to perform experiments on non-primary cardiomyocytes. Single cell calcium imaging measurements led us to identify two subpopulations of cells with different responses to acute stimulation with 10 µM NaHS (Fig. 1). In the majority of cells (60%) NaHS induced a significant decrease of basal $[Ca]_i$ and, in the same population, resting $[Ca]_i$ was higher than that observed in non-sensitive cells. The percentage of responsive cells increased upon pre-activation of voltage-dependent Ca^{2+} channels by high extracellular K$^+$ concentration (Fig. 1B). This evidence suggests than NaHS inhibits VOCCs constitutively open in a subpopulation of non-stimulated H9c2 cells. Accordingly, L-type Ca^{2+} channel blocker Nifedipine induced a larger decrease in resting $[Ca]_i$ in the totality of cells (Fig. 1C-D). Furthermore, resting $[Ca]_i$ levels are higher in H$_2$S-sensitive cells compared to H$_2$S-insensitive and Nifedipine pre-treated cells (Fig. 2A).

Nevertheless, L-type is not the only Ca^{2+} channel type sensitive to H$_2$S. Upon Nifedipine pre-treatment, NaHS was still able to induce $[Ca]_i$ reduction (Fig. 2B and Fig. 3A): this effect was completely prevented by co-incubation with both Nifedipine and Ni^{2+} 100 µM, that blocks all T-type VOCCs (Fig. 3B). There have been previous reports about the T-type channels activation by H$_2$S in neurons [28-30]; on the other hand, Elies et al. reported an inhibitory effect with high doses of NaHS on Cav3.2-overexpressing HEK cells [31]. This is the first preliminary evidence that H$_2$S negatively modulates endogenously expressed T-type Ca^{2+} channels in myoblast cell line.

Interestingly, when both L- and T-type channels are blocked, NaHS promoted a significant increase in $[Ca]_i$. The response was, however, completely prevented in calcium free extracellular medium, suggesting a putative mediation of voltage-independent Ca^{2+} influx (Fig. 3C). These findings confirm our previous results obtained on endothelial cells in which we observed a similar effect due to the activation of voltage-independent calcium channels [32].

At least two different, and possibly coexisting mechanisms, could be responsible for this calcium flux through the plasma membrane of H9c2: the activation of voltage independent calcium-permeable channels as well as a modulation of sodium-calcium exchangers activity.

Among voltage-independent calcium channels, some members of transient receptor potential (TRP) channel family are expressed and functional in H9c2 cells and are proposed to play a role in cardiac hypertrophy and arrhythmia [33-35].

The other possible mechanism underlying H$_2$S-induced calcium inflow can bypass the activation of calcium channels and involve a role for exchangers. In intact endothelium of excised rat aorta, H$_2$S triggers a Ca^{2+} influx driven by the reverse-mode (3Na$^+$ out / 1Ca^{2+} in) of the sodium-calcium exchanger (NCX) and by K$_{ATP}$ channels [21]. It could be interesting to investigate a similar role of NCX in H9c2 cells.

While the functional role of T-type inhibition in H9c2 cells remains unknown, the negative regulation of L-type channels by H$_2$S appears to be involved in cytoprotection against oxidative stress. Indeed, H$_2$S donor and Nifedipine are both effective to prevent H9c2 death induced by H$_2$O$_2$ (Fig. 4). Oxidation by peroxide leads to an irreversible $[Ca]_i$ increase significantly prevented by either NaHS or Nifedipine pre-treatment (Fig. 5). These data

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strongly suggest that the inhibition of L-type channels by H₂S could help H9c2 to maintain low the [Ca]ᵢ levels during oxidative stress induced by H₂O₂, eventually improving cell survival. It is worth noting that L-type H₂S-sensitive channels are not restricted to cardiovascular system and could play different roles, as suggested by the evidence that the same inhibitory effect has been previously described in mouse pancreatic beta cells, affecting insulin secretion [22]. Moreover, a tissue-specific regulation of L-type calcium channels is likely to occur. Indeed, H₂S activates these channels in the nervous system [30, 36], while in other tissues (e.g. cardiovascular system or pancreatic cells) it exerts a negative regulation [15].

A number of questions, beyond the aim of the present study, remain open and deserve more detailed investigation. Here the conclusions are based on calcium imaging and pharmacological approaches. As such, the biophysical and molecular properties of L- and T-type channels in H9c2 cells, as well as their modulation by H₂S should be analyzed by electrophysiological (patch clamp) techniques to strengthen and extend our results.

Finally, this work unveils the ability of H₂S to affect, directly or indirectly, the function of several key channels in H9c2 cells, suggesting its housekeeping and multifunctional role in cell signaling (for a schematic overview see Fig. 6).

Acknowledgements

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