European Journal of Pharmacology 654 (2011) 60–67

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Cardiovascular Pharmacology

Sodium hydrosulfide improves the protective potential of the cardioplegic histidine buffer solution

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ARTICLE INFO

Article history:
Received 14 August 2010
Received in revised form 18 November 2010
Accepted 11 December 2010
Available online 24 December 2010

Keywords:
Hydrogen sulfide
Cardioplegic solution
Ischemia/reperfusion
Histidine buffer

ABSTRACT

Since H_2S has an emerging role as a cardioprotector, we hypothesized that NaHS addition to the new cardioplegic histidine buffer solution (HBS) could improve its cardioprotective potential. Male Wistar–Han rat hearts were divided in 4 groups: i) control, ii) perfusion control (perfusion only), iii) 6 h ischemia in HBS or in a modified-HBS with 100 μM of NaHS, a H_2S donor, (HBSM) and iv) as iii followed by 30 min reperfusion. During ischemia, aliquots of the cardioplegic solution were collected for NMR analysis. Heart mitochondria respiration and transmembrane potential were measured after ischemia or after ischemia followed by reperfusion. Proteins involved in the apoptotic signaling pathway were also quantified in both mitochondrial and tissue samples. Cardiac mechanic performance was evaluated by measuring the heart rate and the left ventricular pressure. In HBSM-preserved hearts, a) glucose consumption increased as well as lactate and alanine production during ischemia, b) heart mitochondria presented an improved phosphorylative efficiency, including decreased phosphorylative lag phase for complex I and complex II substrates, c) mitochondrial and tissue p53, Bax and caspase-9 were lower and d) there was a more positive atrial chronotropic response than in HBS-preserved hearts. We concluded that the addition of NaHS to HBS enhances glycolysis during ischemia, decreases mitochondrial dysfunction, especially by preserving the phosphorylative system, prevents apoptosis and during ischemia/reperfusion.

1. Introduction

During cardiac transplantation procedures, the organ is maintained in cold ischemia (4 °C) and then introduced in the recipient. During the process, lethal injury resulting from apoptosis or necrosis may occur as a result of the sudden reflow of oxygen (Gottlieb et al., 1994).

We have previously described (Alves et al., 2009) that Histidine buffer solution (HBS) presents itself as a valid alternative to the clinically used Celsior solution due to its superior role in the protection of mitochondrial function during ischemia/reperfusion. We have also described that rat male hearts preserved in HBS were more susceptible to protein oxidative damage than hearts from female animals.

In order to increase the effectiveness of HBS as a cardioplegic agent, it is possible to incorporate additives, being hydrogen sulfide (H_2S) a strong candidate. Clear evidence shows that H_2S is the third endogenous signaling gasotransmitter, besides nitric oxide and carbon monoxide (Wang, 2002). H_2S is produced endogenously from L-cysteine and is proposed to have several physiological functions. Tissues such as the brain (Abe and Kimura, 1996) and the heart (Geng et al., 2004), produce H_2S by tissue-specific enzymes. In Sprague–Dawley rats serum, a H_2S concentration of 48 μM was already detected (Zhao et al., 2001) while in human serum the detected concentration was approximately 52 μM (Jiang et al., 2005). It was reported that mice breathing H_2S-enriched gas suffered a 90% decrease in their metabolism and entered a 'suspended animation' process, which was suggested as a possible mechanism of myocardial protection during surgery (Blackstone et al., 2005). It was also reported that exogenous H_2S is protective against cardiac ischemia/reperfusion injury (Johansen et al., 2006) and that NaHS, an H_2S donor, attenuated myocardial injury and improved myocyte survival (Bian et al., 2006). In an *in vivo* model of myocardial ischemia/reperfusion injury, both NaHS (Pan et al., 2009; Sivarajah et al., 2006) and H_2S (Elrod et al., 2007) decreased myocardial infarct size. NaHS also reduced arrhythmias in hearts subjected to ischemia/reperfusion, improved myocyte survival (Pan et al., 2006), attenuated myocardial ischemic injury and increased the hemodynamic recovery rate (Hu et al., 2007). In human derived dopaminergic neuroblastoma (SH-SY5Y), NaHS suppressed apoptotic cellular death by preventing a decrease in Bcl-2/Bax levels, mitochondrial membrane potential ($\Delta\Psi_m$) dissipation, cytochrome c release, caspase-9/3 activation and poly(ADP-ribose) polymerase cleavage (Hu et al., 2009). In a rat model of cardiac

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doi:10.1016/j.ejphar.2010.12.010

ischemia/reperfusion, H_2S treatment reduced Bax translocation into mitochondria and caspase-3 activation (Yao et al., 2010).

Given the cardioprotective potential of H_2S, we hypothesized that the addition of NaHS to HBS, a proposed novel cardioplegic solution (Takeuchi et al., 1999a,b), may improve its cardioprotective potential. To confirm this, we compared the ability of regular HBS and modified HBS (HBSM) to modulate energy metabolism, protect heart mitochondria and prevent mitochondrial accumulation of Bax and p53 during ischemia and ischemia/reperfusion.

2. Materials and methods

2.1. Chemicals

Ketamine was purchased from Merial (Lyon, France), chlorpromazine from Aventis (Lisbon, Portugal), Celsior from IMTIX SANGSTAT (Lyon, France) and D_2O (99.9%) from Cambridge Isotope Laboratories (Cambridge, MA, USA). All other reagents were of analytical grade and purchased from Sigma-Aldrich (Roedermark, Germany).

2.2. Animals

Male Wistar–Han rats (weighting 260 ± 22 g) were housed in our credited animal colony (Laboratory Research Center, University of Coimbra). Animals were group-housed in type III-H cages (Tecniplast, Italy) and maintained in specific environmental requirements (22 °C, 45–65% humidity, 15–20 changes/h ventilation, 12 h artificial light/dark cycle, noise level <55 dB) with free access to standard rodent food (4RF21 GLP, Mucedola, Italy) and water.

All experiments comply with the "Guide for the Care and Use of Laboratory Animals"; published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.3. Ischemia protocol and study groups

Ischemia was performed according to procedures already described (Ackemann et al., 2002; Alves et al., 2009; Jahania et al., 1999). Briefly, after intraperitoneal anesthesia, using ketamine (50 mg/kg) plus chlorpromazine (10 mg/kg) (Ebrahimi et al., 2006), an incision was made on the abdominal area, the thorax was opened, the heart rapidly excised and carefully washed in 10 ml of the respective cardioplegic solution to remove blood from the coronaries and to avoid the formation of blood clots. Finally, the hearts were placed at 4 °C in a conical tube with 10 ml of ice-cold cardioplegic solution and kept immersed for 360 min. Hearts from control group were not subjected to ischemia nor perfusion and in the perfusion control group (Ctrl_P), the hearts were excised and immediately perfused (no ischemia).

The hearts submitted to ischemia were divided in two major groups according to the cardioplegic solution used and subjected to 360 min ischemia followed by 30 min perfusion. The cardioplegic solutions used were a regular HBS or a modified-HBS (HBSM). HBS (in mM, NaCl, 80; KCl, 22.5; $MgSO_4$, 6; $CaCl_2$, 0.1; Na_3PO_4, 2.5; histidine, 100; mannitol, 20; glucose, 11; adenosine 5; lidocain 100 mg/l; insulin 10 UI/l; pH = 7.8) and HBSM (as HBS plus addition of 100 μM of NaHS) were prepared in our laboratory and then filtered (0.2 μm, Millipore) before use.

2.4. Perfusion protocols

A Langendorff perfusion system was used for heart perfusion. Hearts were perfused at constant pressure, 100 cm H_2O, for 30 min at 37 °C with a Krebs–Henseleit solution prepared in our laboratory (in mM: NaCl, 118; KCl, 4.4; $MgSO_4$, 1.2; $CaCl_2$, 1.2; $NaHCO_3$, 25; lactate, 1.2; pyruvate, 0.12; glucose, 5.5; octanoate, 0.3; pH = 7.6) gasified with 95% O_2 and 5% CO_2. For the perfusion control (Ctrl_P), hearts were immediately cannulated after sacrifice and perfused for 30 min. After the 360 min ischemia in the different cardioplegic solutions (HBS or HBSM) the hearts were also cannulated via aorta and perfused for 30 min.

During perfusion, the effluent from the right ventricle was collected in pre-determined time points to measure coronary flow (ml/min). Left ventricular pressure and heart rate were monitored throughout the perfusion using a water-filled balloon placed in the left ventricle and digitally recorded using WinDaq DI-720 recording software (DataQ Instruments, Akron, OH). The hemodynamic parameters were converted into percentage of the perfusion control to describe the relative changes in cardiac function. After the reperfusion period, the hearts were processed for mitochondrial isolation and homogenization for Western Blot analysis.

2.5. Isolation of cardiac mitochondria

Heart mitochondria were isolated according to standard procedures in our laboratory (Alves et al., 2009). The pellet was resuspended in 500 μl of washing medium (in mM: saccharose, 250; Hepes, 10; pH = 7.4). Protein was quantified by biuret method using BSA as standard.

2.6. Measurements of mitochondrial transmembrane potential ($\Delta\Psi$) and oxygen consumption

The mitochondrial transmembrane electric potential ($\Delta\Psi$) was estimated with a tetraphenylphosphonium (TPP^+) electrode and oxygen consumption of isolated heart mitochondria was monitored polarographically with a Clark oxygen electrode connected to a suitable recorder in 1 ml thermostated closed chamber with magnetic stirring by standard procedures used in our laboratory (Alves et al., 2009). A matrix volume of 1.1 μl/mg protein was assumed. Reactions were carried out at 25 °C in 1 ml standard medium (50 mM saccharose, 100 mM KCl, 10 μM EGTA, 10 mM Tris and 1 mM KH_2PO_4, pH = 7.4) supplemented with 3 μM TPP^+ and 0.5 mg of heart mitochondria. Energized heart mitochondria were obtained using complex-specific substrates, 10 mM of glutamate/malate for complex I-dependent energization (CxI) or 8 mM succinate (plus 4 μM rotenone) to energize mitochondria through complex II (CxII). For simplification, respiratory activity caused by glutamate/malate addition is referred as Complex I (CxI) and the respiratory activity observed after succinate addition plus rotenone is referred as Complex II (CxII). After energization, when the potential reached a stable value, 250 nmol of ADP were added to induce state 3 respiration. After state 4 was achieved, 2 μg of oligomycin were added to assess the contribution of proton passive leak through the ATP synthase. Finally, 0.5 μM of Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) were added to induce uncoupled respiration. The ADP/Oxygen ratio is calculated as nmol of ADP used to produce ATP per natoms of oxygen consumed and will be hereafter described as phosphorylation efficiency index. The respiratory control ratio (RCR) is determined from the ratio between state 3 and state 4 respirations.

2.7. Western blot analysis

Frozen mitochondrial or whole heart tissue preparations were homogenized in lysis buffer (1 M urea, 10 mM Tris, 2% SDS, pH = 7.5) and heated for 10 min at 60 °C.

Aliquots of 50 μg protein were fractionated in 12% polyacrylamide gels and electrophoresis was carried out for 1 h with 30 mA per gel. The separated proteins were afterwards transferred to polyvinylidene difluoride membranes and blocked for 1 h in a 5% non-fat milk solution at 37 °C. The membranes were then incubated overnight at 4 °C with goat anti-p53 (1:1000, Santa Cruz Biotechnology,

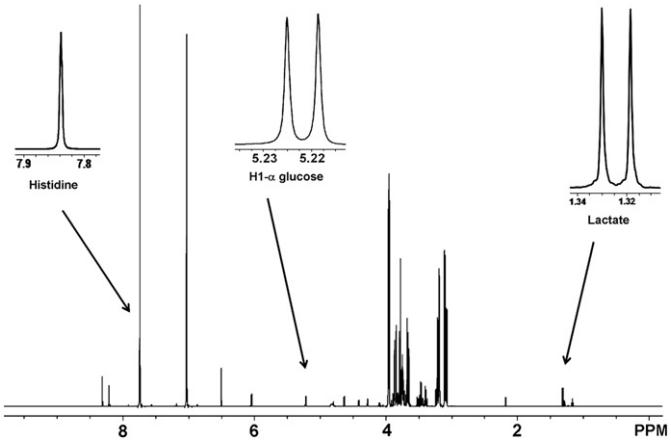

Fig. 1. Representative ^{1}H NMR spectrum attained for HBS-preserved hearts and expansion of multiplets of lactate (1.33 ppm), H1-α-glucose (5.22 ppm) and histidine (7.80 ppm). The relative areas of ^{1}H NMR resonances were quantified using the curve-fitting routine supplied with the NUTSpro™ NMR spectral analysis program (Acorn, NMR Inc, Fremont, CA).

Heidelberg, Germany, Sc 6243) or rabbit anti-Bax (1:5000, Cell Signaling, Massachusetts, USA, # 2772). As protein loading control for mitochondrial fraction and heart tissues we used mouse anti-actin (1:200, Sigma, Roedermark, Germany, A 5441). The immuno-reactive proteins were detected separately and visualized with rabbit anti-goat IgG-AP (1:5000, Santa Cruz Biotechnology, Heidelberg, Germany, Sc 2771), goat anti-rabbit IgG-AP (1:5000, Santa Cruz Biotechnology, Heidelberg, Germany, Sc 2007) or goat anti-mouse IgG-AP (1:5000, Santa Cruz Biotechnology, Heidelberg, Germany, Sc 2008). Membranes were reacted with ECF detection system (GE Healthcare, Weßling, Germany) and read with the Versa Doc imagining system (Bio-Rad, Hemel Hempstead, UK). Densities from each band were obtained with Quantity One Software (Bio-Rad, Hemel Hempstead, UK) according to standard methods. The band density obtained was then divided by the respective actin band density and expressed in percentage versus the control group.

2.8. NMR spectroscopy

During heart preservation, 160 µl of the preservation solution were collected at 30, 60, 120, 180, 240, 300 and 360 min, for NMR analysis. To each sample were added 40 µl of a 10 mM sodium fumarate

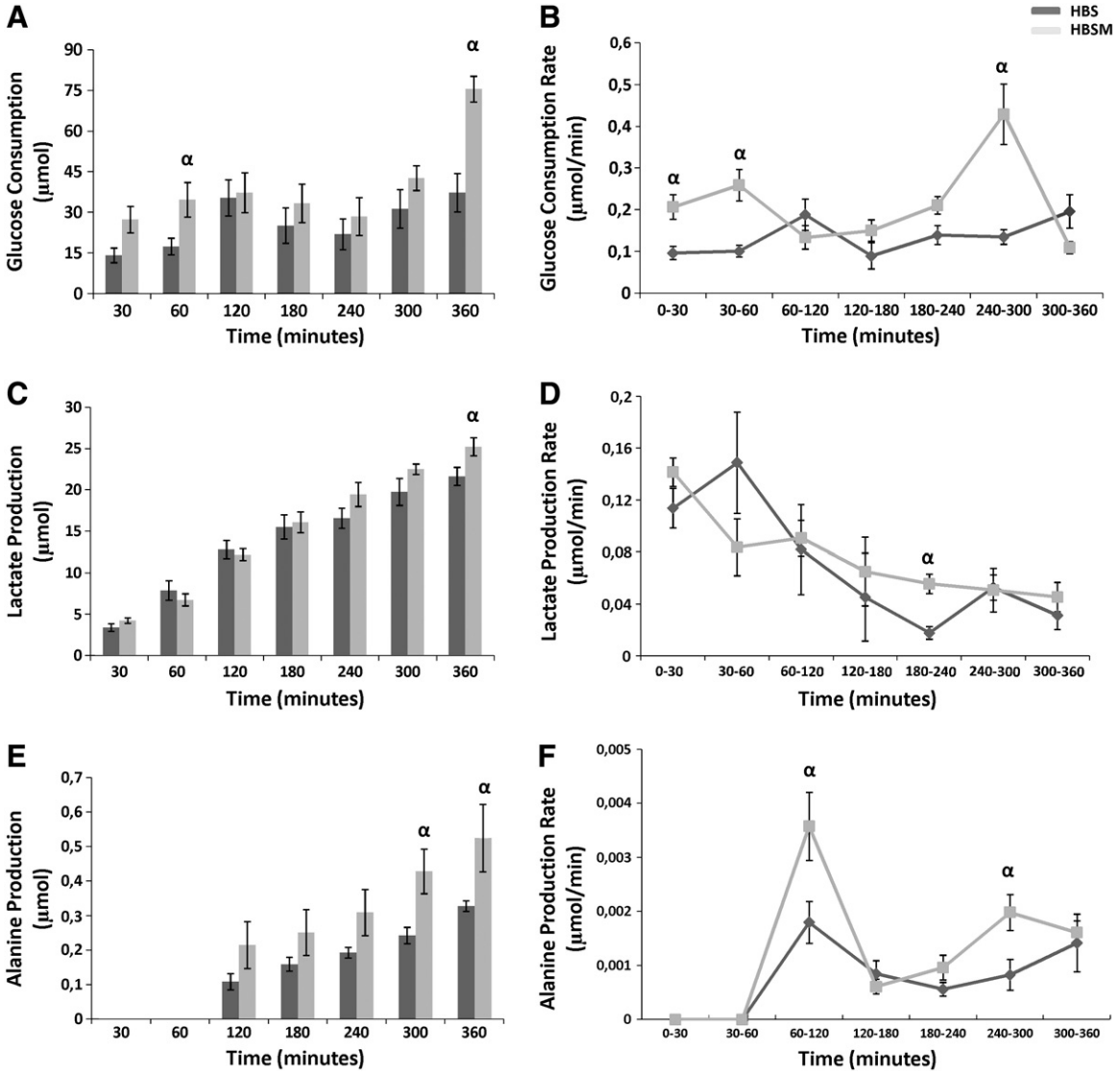

Fig. 2. Glucose consumption (A), glucose consumption rate (panel B), lactate production (C), lactate production rate (D), alanine production (E) and alanine production rate (F) during 360 min heart preservation in HBS (gray) and HBSM (light gray). Significantly different results ($P<0.05$) are as indicated: α — versus HBS ($n=5$ for each condition).

solution in 99.9% D_2O. ^1H NMR spectra of the collected samples were acquired at 14.1 T, 25 °C, using a Varian 600 MHz spectrometer equipped with a 3 mm indirect detection probe with z-gradient (Varian, Palo Alto, CA). Solvent-suppressed ^1H NMR spectra were acquired with 6 kHz sweep width, using 14 s delay for allowing total proton relaxation, 3 s water pre-saturation, 45° pulse angle, 3.5 s acquisition time, and at least 64 scans.

^1H NMR spectroscopy was performed to determine lactate production, glucose consumption and variation in other substances such as alanine and histidine, during the 360 min of ischemia. Sodium fumarate (final concentration: 2 mM) was used as an internal reference (6.50 ppm). The following metabolites were determined whenever present: lactate, doublet located at 1.33 ppm; alanine, doublet at 1.45 ppm, H1-α glucose doublet at 5.22 ppm; and histidine, singlet at 7.80 ppm.

The relative areas of ^1H NMR resonances were quantified using the curve-fitting routine supplied with the NUTSpro™ NMR spectral analysis program (Acorn, NMR Inc, Fremont, CA).

2.9. Statistical analysis

The results are presented as mean ± S.E.M (n = 5 for each condition). Statistical comparison between selected experimental groups and/or subgroups was performed by using a two-way ANOVA (GraphPad Software Inc.). $P<0.05$ was considered significant.

3. Results

3.1. The addition of NaHS to HBS enhances glycolysis during ischemia

Whenever present, the following metabolites were determined in the NMR spectra: lactate, doublet located at 1.33 ppm; alanine, doublet at 1.45 ppm, H1-α glucose doublet at 5.22 ppm; and histidine, singlet at 7.80 ppm (Fig. 1). Hearts preserved in HBSM consumed significantly more glucose (76 ± 5 μmol) than those preserved in HBS (37 ± 7 μmol) after 360 min of ischemia. During the first 60 min, glucose consumption was already significantly higher in hearts preserved in HBSM than in those preserved in HBS (Fig. 2A). Between 30 and 60 min of ischemia, hearts preserved in HBSM presented significantly higher glucose consumption rate (0.26 ± 0.03 μmol/min) than those preserved in HBS (0.10 ± 0.01 μmol/min). The major difference was noted between 240 and 300 min of ischemia. During this time period, glucose consumption rate was 0.14 ± 0.02 and 0.43 ± 0.07 μmol/min in hearts preserved in HBSM and HBS, respectively (Fig. 2B).

Concerning lactate production, hearts preserved in HBSM produced significantly more (25 ± 1 μmol) than those preserved in HBS (22 ± 1 μmol) (Fig. 2C). Lactate production rate was very similar in hearts preserved in HBSM and HBS except between 180 and 240 min of elapsed time. During this period, lactate production rate was significantly higher in hearts preserved in HBSM (Fig. 2D).

Alanine production was only observed after 120 min of ischemia. HBSM-preserved hearts produced significantly more alanine (0.53 ± 0.09 μmol) after 360 min than HBS-preserved hearts (0.33 ± 0.02 μmol) (Fig. 2E). Alanine production rate was also significantly ($P<0.05$) different for some of the time periods measured. Between 60 and 120 min, hearts preserved in HBSM produced alanine at a rate of 0.0018 ± 0.0003 μmol/min while hearts preserved in HBS produced 0.0036 ± 0.0004 μmol/min (Fig. 2F).

3.2. The addition of NaHS to HBS protects the mitochondrial phosphorylative system after ischemia/reperfusion

One particular aspect of cardiomyocyte injury during ischemia/reperfusion is the disruption of mitochondrial function. Several endpoints of mitochondrial function were evaluated, including mitochondrial respiration (state 3, state 4, the respiratory control and phosphorylation efficiency index, state 4 in the presence of oligomycin and uncoupled respiration), as well as variations in the $\Delta\Psi$ associated to ATP synthesis (maximum-attained $\Delta\Psi$, ADP-induced depolarization and phosphorylative lag phase). Perfusion (Ctrl_P) was not able, per se, to induce major alterations in both heart mitochondria complex I (CxI) and complex II (CxII)-sustained mitochondrial respiration and $\Delta\Psi$. After 6 h ischemia followed by 30 min perfusion, clear differences between the two solutions were found in the phosphorylation efficiency index and in the phosphorylative lag phase which is defined as half the time required for membrane repolarization after ADP phosphorylation. The phosphorylation efficiency index, or ADP/oxygen ratio, reveals the relationship between ADP usage for ATP synthesis and oxygen consumption, decreased in CxI from 2.6 ± 0.2 in the control group (no ischemia, no perfusion) to 1.5 ± 0.1 in HBS-preserved hearts and 2.0 ± 0.1 in the HBSM-preserved hearts (Fig. 3A). The phosphorylative lag phase was 29 ± 4 s in the control and significantly increased to 86 ± 7 s in HBSM-preserved hearts. Interestingly, when hearts were preserved in HBS, an absolute value to the phosphorylative lag phase could not be

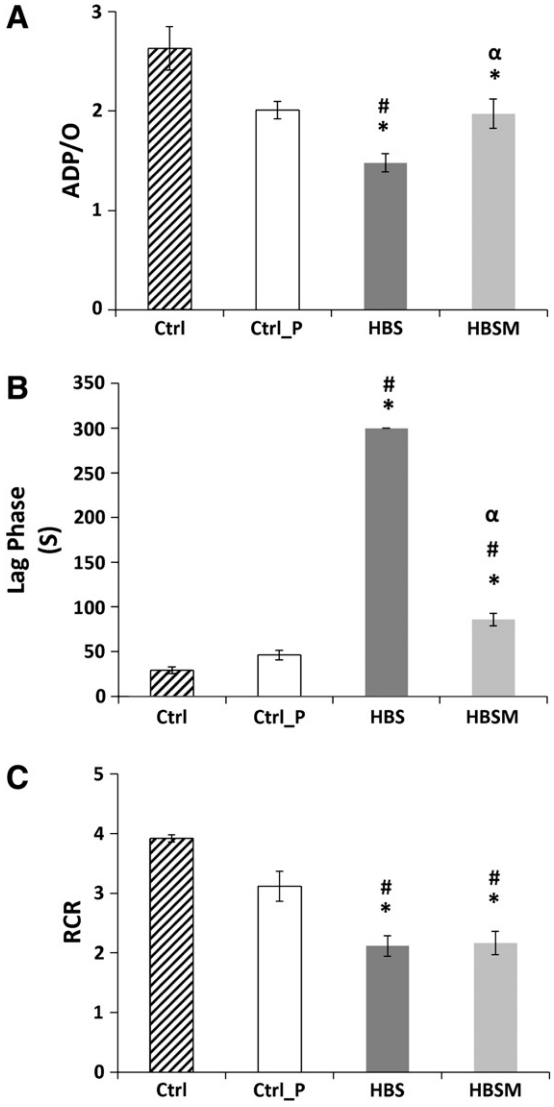

Fig. 3. Phosphorylation efficiency index (ADP/O) (A), phosphorylative lag phase (B) and respiratory control rate (RCR) (C) measured for complex I substrates (CxI) in the four experimental groups: control (Ctrl), perfusion control (Ctrl_P), heart preservation in HBS and 30 min perfusion (HBS) and after heart preservation in HBSM and 30 min perfusion (HBSM). Significantly different results ($P<0.05$) are as indicated: * — versus control; # — versus perfusion control and α — versus HBS. (n = 5 for each condition).

determined (Fig. 3B). After 300 s, the ΔΨ still did not recover to its initial value and in some cases, further depolarization occurred. The respiratory control ratio, which is defined as the ratio between state 3 and state 4 respiration, significantly decreased from 3.9 ± 0.1 in heart mitochondria from the control group to 2.1 ± in heart mitochondria from hearts preserved in HBSM and HBS (Fig. 3C).

Using complex II substrates (CxII), the phosphorylation efficiency index significantly decreased from 1.6 ± 0.1 in the control group to 1.0 ± 0.1 and 1.2 ± 0.1 in HBSM and HBS-preserved hearts, respectively (Fig. 4A). The phosphorylative lag phase significantly increased from 37 ± 2 s in the control to 130 ± 4 s in HBSM-preserved hearts. Similar to what happened with CxI, the lag phase was not possible to determine in HBS-preserved hearts even after 300 s (Fig. 4B). The respiratory control ratio significantly decreased from 2.0 ± 0.1 in the control group to 1.5 ± 0.1 and 1.2 ± 0.1 in HBSM and HBS-preserved hearts, respectively (Fig. 4C).

Concerning mitochondrial transmembrane potential measurements, ΔΨmax and ADP-induced depolarization were significantly decreased after 6 h ischemia and perfusion; however, the protection afforded by HBSM and HBS was similar and no significant differences between both cardioplegic solutions were noted. The perfusion (Ctrl_P) was not able, per se, to induce significant differences in mitochondrial membrane potential values when compared with the control (Table 1).

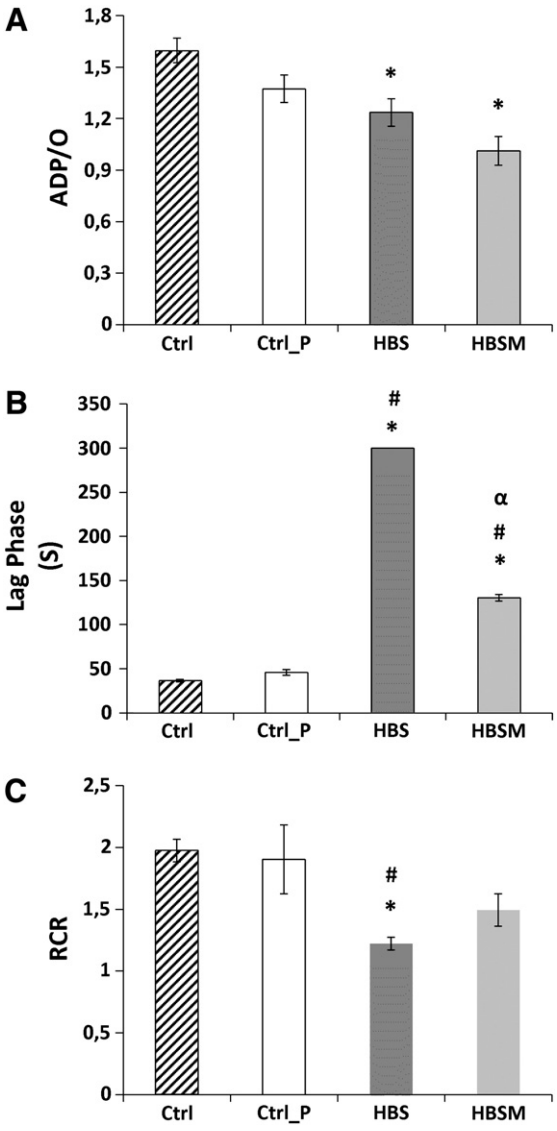

Fig. 4. Phosphorylation efficiency index (ADP/O) (A), phosphorylative lag phase (B) and respiratory control ratio (RCR) (C) measured for complex II substrates (CxII) in the four experimental groups: control (Ctrl), perfusion control (Ctrl_P), heart preservation in HBS and 30 min perfusion (HBS) and after heart preservation in HBSM and 30 min perfusion (HBSM). Significantly different results ($P<0.05$) are as indicated: * — versus control; # — versus perfusion control and α — versus HBS. (n = 5 for each condition).

3.3. Mitochondrial accumulation of Bax and p53 is decreased when NaHS is added to HBS

In mitochondrial and tissue samples, heart perfusion was not able, per se, to induce any alterations in Bax or p53 protein levels relatively to the control. Interestingly, mitochondrial p53 and Bax content significantly decreased to 55 ± 7 and 47 ± 5% of the control value in HBSM-preserved hearts. On the contrary, HBS-preserved hearts presented a significant increase in Bax protein levels (Fig. 5A).

In whole cardiac tissue, Bax protein levels significantly decreased to 56 ± 11 and 89 ± 5% of the control value in HBSM and HBS-preserved hearts, respectively (Fig. 5B). In order to assess mitochondrial-dependent apoptotic cell death, the active fragment of caspase-9 (10 kDa) was evaluated by Western Blot. HBS-preserved hearts presented a significant increase in cleaved caspase-9 protein levels (166 ± 11% of the control value) while HBSM-preserved hearts showed a lower value for the active caspase-9 fragment in 100 ± 7% (Fig. 5B).

3.4. The addition of NaHS to HBS increases heart rate but not left ventricular pressure

Cardiac mechanic performance was evaluated by measuring coronary flow, heart rate and also left ventricular pressure. The coronary flow throughout the perfusion did not significantly change and only slight variations between 9.3 ± 1.5 and 11.2 ± 1.8 ml/min were observed between the different experimental groups (data not shown). Heart rate was distinct between HBSM and HBS-preserved hearts. HBSM-preserved hearts presented a significantly higher heart rate. The heart rate for HBS-preserved hearts was 78 ± 9% of the Ctrl_P value while HBSM-preserved hearts increased this parameter to 124 ± 12% of the Ctrl_P value. The left ventricular developed pressure and left ventricular end-diastolic pressure remained unaltered in both HBS and HBS-preserved hearts (Fig. 6).

4. Discussion

Based on its endogenous metabolism and physiological functions, H_2S is well positioned in the family of endogenous gaseous transmitters, by acting as a signaling molecule of the inflammatory, nervous and also in the cardiovascular system where it regulates vascular tone and cardiac work.

Table 1
Maximum mitochondrial transmembrane electric potential (ΔΨmax) and ADP-induced depolarization in heart mitochondria from the control (Ctrl), perfusion control (Ctrl_P) or from hearts preserved during 6 h in HBS or HBSM. Significantly different results ($P<0.05$) are as indicated: a — versus control; b — versus perfusion control (n = 5 for each condition).

	Complex I		Complex II	
	ΔΨmax (−mV)	ADP-induced depolarization (−mV)	ΔΨPmax (−mV)	ADP-induced depolarization (−mV)
Ctrl	202 ± 2	34 ± 2	204 ± 1	37 ± 2
Ctrl_P	200 ± 3	30 ± 3	200 ± 2	36 ± 2
HBS	185 ± 5[a,b]	31 ± 3	177 ± 5[a,b]	21 ± 2[a,b]
HBSM	189 ± 1[a,b]	26 ± 2	186 ± 2[a,b]	22 ± 2[a,b]

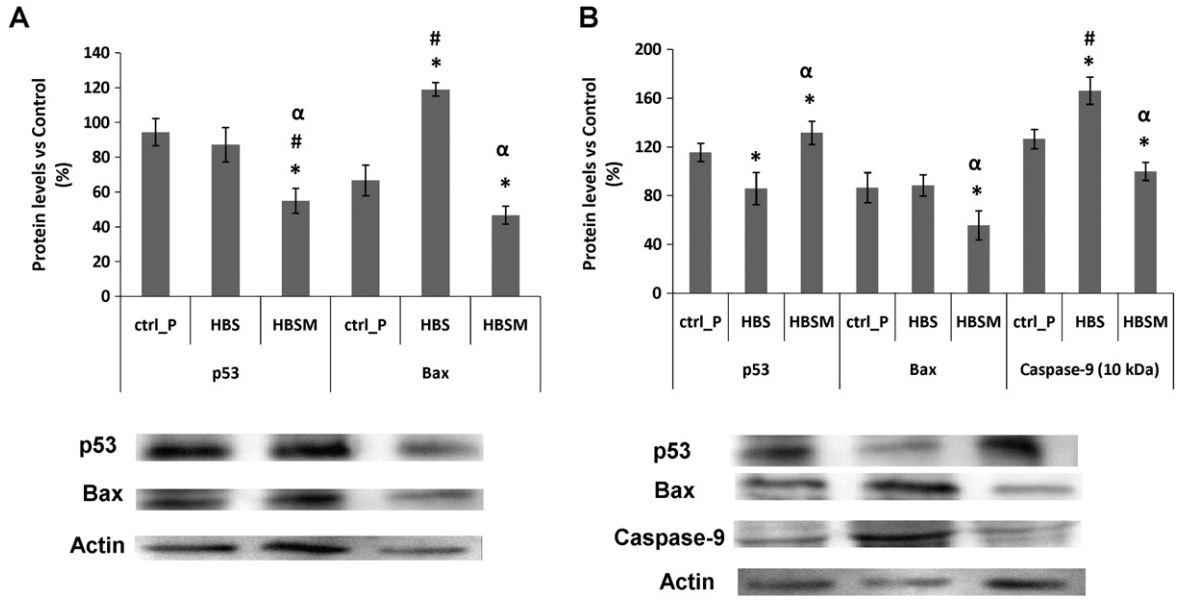

Fig. 5. Mitochondrial p53 and Bax content in hearts subjected to 30 min perfusion only (Ctrl_P) or preserved during 6 h in HBS or HBSM prior to perfusion (A). Protein levels of Bax and active caspase-9 fragment in tissue samples from hearts subjected only to 30 min perfusion (Ctrl_P) or preserved during 6 h in HBS or HBSM prior to perfusion (B). Protein levels are normalized for actin content and expressed in percentage of the control group. Significantly different results ($P<0.05$) are as indicated: * — versus control; # — versus perfusion control and α — versus HBS. ($n=5$ for each condition).

In the present work, NaHS was used as a source of H_2S because: a) NaHS dissociates to Na^+ and HS^- in solution, then HS^- associates with H^+ and produces H_2S (about one-third of the existing is in the undissociated form (H_2S) and the remaining two-thirds as HS^- equilibrium with H_2S) (Beauchamp et al., 1984; Bian et al., 2006; Dombkowski et al., 2004); b) the use of NaHS defines more accurately and reproducibly the concentrations of H_2S in solution; c) the concentration of NaHS used does not change the pH of the medium (Hosoki et al., 1997). So, approximately 33 μM of H_2S, which is within the physiological concentration detected in rat serum (Zhao et al., 2001), is present in solution. Others have already described the advantageous of using NaHS as H_2S donor due to its stability (Ji et al., 2008; Pan et al., 2009; Su et al., 2009).

We found that the addition of NaHS to HBS enhances glucose consumption, as reflected by the increase in lactate and alanine production during 6 h ischemia. Glucose consumption and production rates of lactate and alanine were also higher in HBSM-preserved hearts, at least during some periods. When the heart is subjected to cold ischemia, a shortage of oxygen occurs, which drives anaerobic metabolism and fast glucose uptake increasing glycogenolytic and glycolytic fluxes. As a consequence, glucose metabolism is redirected to lactate and alanine production (Morgan et al., 1961). The reduction of pyruvate into lactate or its conversion into alanine is related with the re-oxidation of cytosolic NADH into NAD^+ and although it has been described that H_2S increases the intracellular NADPH/$NADP^+$ ratio (Deplancke and Gaskins, 2003), our results suggest that the redox state of HBS and HBSM-preserved hearts was similar since the lactate/alanine ratio was identical in both situations. Simon et al. (2008) described that in mechanically-ventilated pigs, the administration of H_2S induced a comparable metabolic response to anesthetized pigs and that H_2S decreased the rate of metabolic expenditure. The same authors also related the decrease in O_2 uptake during exercise, induced by inhaling low-dose gaseous H_2S, with significantly higher blood lactate concentrations (Bhambhani and Singh, 1991; Khan et al., 1990). On the contrary, our results show that when NaHS was added to HBS, the increase in glucose consumption was more significant than the increase in lactate production, which is consistent with an increase in the rate of metabolic expenditure. This may be due to the fact that the concentration of gas acting in organs and tissues in studies when inhaling H_2S is used is much different from the concentration used in this study and most certainly the metabolic response to NaHS is dose-dependent.

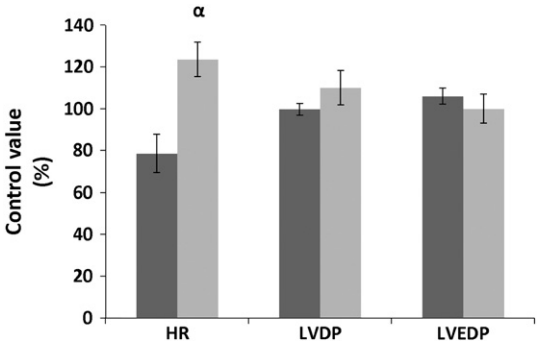

Fig. 6. Heart rate (HR), left ventricular developed pressure (LVDP) and left ventricular end-diastolic pressure (LVEDP) averaged over the 30 min perfusion after 6 h heart preservation in HBS (grey) and HBSM (light grey). The results are presented as percentage (%) of perfusion control value. Absolute values for the perfusion control are 252 ± 18 beats min^{-1} for heart rate, 107 ± 9 mm Hg for the left ventricular developed pressure and 8 ± 1.2 mm Hg for left ventricular end-diastolic pressure. Significantly different results ($P<0.05$) are as indicated: α — versus HBS. ($n=5$ for each condition).

In vivo, H_2S is metabolized via oxidation in mitochondria or methylation in cytosol, and scavenged by metalloproteins, disulfide-containing proteins, thio-S-methyl-transferase, and heme compounds (Wang, 2002). H_2S has been related with the downregulation of transcripts of oxidative phosphorylation proteins (including mitochondrial cytochrome oxidase subunits I, II, III, cytochrome c oxidase subunit IV, and ATP synthase) (Deplancke and Gaskins, 2003). In our experimental conditions, the addition of NaHS to HBS prevented, in some degree, mitochondrial dysfunction associated with our ischemia/reperfusion conditions, via preservation of the mitochondrial phosphorylative system, as seen by the improvements in the phosphorylation efficiency index and phosphorylative lag phase by using complex I and complex II substrates.

Apoptosis of cardiomyocytes is considered to be one major cause for heart failure. Upon caspase activation, cellular death is generally thought to be inevitable. Upon apoptotic stimuli in different organs,

p53 and Bax translocation to mitochondria contributes to mitochondrial membrane disruption and release of apoptotic proteins (Chipuk et al., 2004).

Others have suggested that the cardioprotective effect of NaHS might be secondary to its anti-apoptotic and anti-inflammatory effects which may occur, for example, by attenuating the increase in ischemia/reperfusion-induced caspase 9 activity (Sivarajah et al., 2009). From our results, we concluded that the addition of NaHS to HBS prevented the accumulation of p53 and Bax in mitochondria, which may contribute to decreased mitochondrial membrane permeabilization and apoptotic signaling. Also important was the decrease of caspase-9 active fragment in the tissues of hearts preserved in HBSM. The antiapoptotic effects of H_2S are also associated with an inhibition on mPTP opening, and the decrease of Bax translocation has also been described by others (Yao et al., 2010). NaHS has also been described to decrease the leakage of cytochrome c from mitochondria to cytoplasm, thus inhibiting apoptosis and improving mitochondrial alterations such as swelling, disorganized cristae and attenuation of myofibrillar disorganization (Wang et al., 2011), which agrees with our results showing lower Bax accumulation in mitochondria and lower caspase 9 activation. It was also demonstrated that NaHS decreases caspase 9 activity and decreases the expression of Bcl-2 in isolated cardiomyocytes of hearts subjected *in vivo* to regional myocardial ischemia/reperfusion (Sivarajah et al., 2009).

We also cannot exclude that the antiapoptotic effect and the improvement in mitochondrial function preservation in HBSM-preserved hearts may be mediated by the opening of the mitochondrial K_{ATP} channel. The activation of those channels inhibits oxidative stress-induced apoptosis in isolated cardiomyocytes (Akao et al., 2001). H_2S is, in fact, the first identified gaseous opener of K_{ATP} channels in vascular smooth muscle cells (Zhao et al., 2001) and the opening of the K_{ATP} channels in myocardium is an important endogenous cardioprotective mechanism and a crucial component of cardiac ischemic preconditioning (IPC) (Gross and Fryer, 1999). Others have suggested that the opening of the K_{ATP} could benefit the preservation of isolated hearts (Behling and Malone, 1995; Dorman et al., 1997). Sivarajah et al. (2006) demonstrated that intravenous administration of NaHS in anesthetized rats reduced the myocardial infarct size caused by ischemia/reperfusion and that the protection caused by H_2S was attenuated by 5-hydroxydecanoate (5-HD), a mitochondrial selective K_{ATP} channel antagonist.

The major determinants of myocardial function and viability are the heart rate and left ventricular pressure (King et al., 1995). Our results show that HBS-preserved hearts have lower heart rate when compared with HBSM-preserved hearts. The results suggest that when hearts were preserved in HBSM, the depression in cardiac heart rate is less affected than when the hearts were preserved in standard HBS. However, there was no difference in the left ventricular pressure (LVDP and LVEDP) by HBS or HBSM-preserved hearts. As such, the addition of NaHS to HBS elicited a positive atrial chronotropic response and not a ventricular effect. This interesting observation will certainly deserve more attention in the future. The characterization of left ventricular recuperation after ischemia and ischemia followed by reperfusion in the presence of H_2S also needs further understanding. Our results are consistent with findings from other authors (Zhao and Wang, 2002; Zhao et al., 2001) that described H_2S as a vasorelaxant.

Mitochondrial dysfunction leads to apoptosis which reduces the number of viable cardiomyocytes and contributes to myocardial stunning (Anselmi et al., 2004) which is directly linked to a lower glucose requirement. Our results show that the addition of NaHS to HBS enhances glycolysis during ischemia, decreases mitochondrial dysfunction, especially in what concerns to the phosphorylative system, prevents the apoptotic process and enhances cardiac performance after ischemia/reperfusion.

More studies are needed to better understand the molecular mechanisms behind the protective effect of the addition of H_2S to cardioplegic solutions. The molecular mechanisms by which H_2S induces cardioprotection may open new therapeutic interventions against ischemia/reperfusion injury and help in the development of new and more effective cardioplegic solutions.

Acknowledgment

The Portuguese Foundation for Science and Technology is thanked for financially supporting this study (SFRH/BD/31655/2006 to Marco G. Alves; POCI/SAU-OBS/55802/2004 and PTDC/SAU-OSM/104731/2008 to Rui A. Carvalho).

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