Biomedicine & Pharmacotherapy 97 (2018) 271–279

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

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

Original article

Role of endogenous hydrogen sulfide in cardiac mitochondrial preservation during ischemia reperfusion injury

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ARTICLE INFO

Keywords:
Hydrogen sulfide preconditioning
Myocardial ischemia reperfusion
Propargyl glycine
2,4-Dinitrophenol

ABSTRACT

Cardio-protective effect of hydrogen sulfide (H_2S) against myocardial ischemia reperfusion injury (I/R) via preservation of mitochondria is well documented. But the distinct role of exogenous and endogenous H_2S in cardio-protection and its dependency on functional cardiac mitochondria is not understood. The present study was designed to investigate the role of exogenous H_2S preconditioning on cardiac mitochondrial subpopulation namely interfibrillar (IFM) and subsarcolemmal (SSM), in attenuating I/R injury in an isolated rat heart model in the absence of endogenous H_2S production. The well-known inhibitor of endogenous H_2S production DL-propargylglycine (cystathionine gamma lyase inhibitor) used for this purpose. Our previous studies revealed that NaSH (a H_2S donor) preconditioning at a dose of 20 μM significantly reduced the infarct size and preserved the functional activities of IFM. However, this protective effect significantly declined, when the heart preconditioned with NaSH in the presence of PAG. Apparently, cardiac recovery was improved hemodynamically to a minimal level by increased concentration of NaSH (40 μM). The rat heart perfused with 2, 4 dinitrophenol a mitochondrial uncoupler, before NaSH preconditioning to understand the mitochondrial dependency of exogenous NaSH, we found that cardio-protective effect of NaSH was negated even with higher concentration of H_2S. The result indicates that exogenous NaSH mediated cardio protection depends on functional cardiac mitochondria. However, no functional difference between the subpopulation i.e., interfibrillar and subsarcolemmal mitochondria observed with NaSH preconditioning in the presence of PAG.

1. Introduction

Hydrogen sulfide (H_2S) is well-recognized gasotransmitter of heart along with nitric oxide (NO) and carbon monoxide (CO), capable of modulating numerous physiological processes. H_2S limit the infarct size and preserving the left ventricular (LV) function in both in vivo and in vitro models of myocardial ischemia-reperfusion injury (I/R). The underlined cytoprotective effect of H_2S against myocardial I/R associated with an inhibition of myocardial inflammation, oxidative stress, calcium overload and the preservation of both mitochondrial structure and function of electron transport chain [1–3]. Although long considered as a harmful toxic gas, recently accumulated evidence support the prominent role of H_2S in cardio-protective signaling pathways that converged towards the mitochondria [3].

Endogenous generation of H_2S is mediated by the enzymes cystathionine gamma lyase (CSE) and cystathionine beta synthase (CBS) from L-cysteine in the mammalian body. The rat myocardium majorly comprises of increased expression of CSE mRNA, indicating a considerable amount of H_2S generation by this enzyme [4]. A decline in the level of H_2S in both plasma and the myocardium has been reported in an isoproterenol-induced myocardial infarction in the rat, emphasizing the key role of endogenous production of H_2S in maintaining the cardiac physiology [5]. Further studies by Elrod and his associates demonstrated that over-expression of CSE could reduce the myocardial I/R injury by reducing myocardial inflammation and preserving mitochondrial function [6].

In addition to CBS and CSE, there exist two mitochondrial sources of H_2S production by cysteine aminotransferase and 3-mercaptopyruvate sulfurtransferase [7]. The activity of these enzymes is suppressed by calcium overload and oxidative stress, which are the main pathological events of I/R injury, during which a lower H_2S production has been reported [8]. At low concentrations, H_2S (< 10 μM) increases mitochondrial O_2 consumption and ATP production while at higher concentrations (1 mM), the opposite effects are observed due to complex-IV inhibition [7,9]. Further, studies have established that transient inhibitory effects of sulfide on mitochondrial electron transport induces

Abbreviations: I/R, ischemia reperfusion injury; IFM, interfibrillar mitochondria; SSM, subsarcolemmal mitochondria; DNP, 2,4-dinitrophenol; CSE, cystathionine gamma lyase; PAG, DL-propargyl glycine

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http://dx.doi.org/10.1016/j.biopha.2017.10.118
Received 13 June 2017; Received in revised form 20 October 2017; Accepted 21 October 2017

cytoprotective effects through mitochondria-mediated stress signaling, by retaining a "suspended-animation" like state that protects against myocardial I/R injury [10,11]. Our previous study results revealed H_2S mediated cardio-protection is via preservation of the interfibrillar mitochondrial (IFM) subpopulation activity in the I/R rat heart [3]. Therefore, in the present study, we aimed to find the role of NaSH mediated cardio-protection in the absence of endogenous H_2S and its effect on the cardiac mitochondrial subpopulation.

2. Materials and methods

2.1. Animals

Male Wistar rats (200–250 g) housed in polycarbonate cages were used for the study with prior approval form the institutional animal ethical committee at SASTRA University (No 230/SASTRA/IAEC/RPP). The experimental protocols were conducted as per the guidelines of the Committee for the Purpose of Conduct and Supervision of Experiments on Animals (CPCSEA), Government of India. An acclimatized for one week with standard laboratory diet and tap drinking water was provided before the start of the experiment.

2.2. Isolated heart perfusion protocol

Rats were injected with heparin 500 IU intraperitoneal 30 min before anaesthesia to prevent coagulation of blood while excision of the heart and then anaesthetized by administrating thiopentone sodium (60 mg/kg of body weight). The heart was excised and mounted on the Langendorff apparatus (AD instruments, Australia), which works retrograde, through the aorta by perfusing KH (Kreb's Hensleit) buffer containing NaCl (118.5 mM), KCl (5.8 mM), $NaHCO_3$ (25 mM), KH_2PO_4 (1.2 mM), $MgSO_4$ (1.2 mM), glucose (11 mM), and $CaCl_2$ (2.5 mM) at pH = 7.4, maintained at 37 °C and bubbled with carbogen gas (5% CO_2, and 95% O_2). All hearts were initially stabilized for 20 min at a constant pressure of 70 mmHg.

2.3. Experimental design

Animals were assigned to eight experimental groups consisting of 6 rats each as mentioned below

1. *Sham*: Rats hearts were perfused with KH buffer for 110 min, without inducing injury
2. *Ischemia-Reperfusion* (*I/R*): hearts were perfused with KH buffer and subjected to 30 min global ischemia by stopping the buffer flow followed by 60 min reperfusion
3. *H_2S preconditioning* (*HIPC*): NaSH (20 μM) was perfused along with KH buffer for 15 min before global ischemia induction (30 min) followed by 60 min reperfusion
4. *PAG control*: Hearts were perfused with DL-propargyl glycine (0.2 μM) for 15 min before they were subjected to sham perfusion as per group 1.
5. *PAG + HIPC*: PAG perfusion as per group 5 was followed by NaSH (20 μM or 40 μM) perfusion for 15 min before global ischemia induction, followed by 60 min reperfusion.

2.4. Hemodynamic measurement

A latex balloon was inserted into the left ventricle of the heart to monitor the cardiac ventricular hemodynamics. The balloon was connected to a pressure transducer and the signal was recorded using PowerLab 8/35 data acquisition system and the following hemodynamic parameters were read during the entire duration of each experiment and evaluated using LabChart pro from AD instruments Ltd, Australia; left ventricular end diastolic pressure (LVEDP) in mmHg, developed pressure (LVDP) in mmHg, heart rate (HR) in beats per minute (bpm), and rate pressure product (RPP = HR × DP).

Table 1
Hemodynamics measurement at the end of reperfusion. Data are represented as mean ± SD. * P < 0.05 vs Sham; $ – P < 0.05 vs I/R. LVEDP- Left ventricular end diastolic pressure; LVDP- Left ventricular developed pressure; RPP- Rate pressure product.

Groups (n = 6/ group)	LVEDP (mm Hg)	LVDP (mm Hg)	RPP (mm Hg*Sec*10^3)
Sham	7 ± 2	99 ± 4	96 ± 2
I/R	46 ± 3*	43 ± 3*	33 ± 2*
HIPC (20 μM)	18 ± 3$	97 ± 3$	89 ± 2$
PAG control	8 ± 3	97 ± 2	93 ± 1
PAG + HIPC (20 μM)	27 ± 2	51 ± 3	56 ± 3
PAG + HIPC (40 μM)	21 ± 2	64 ± 2	75 ± 2

Table 2
Infarct size measured at the end of reperfusion using TTC staining. Data are represented as mean ± SD. * P < 0.05 vs Sham; $ P < 0.05 vs I/R.

Groups	Infarct size (% of total heart)
Sham	4 ± 0.8
I/R	28 ± 0.9*
HIPC (20 μM)	8 ± 0.5$
PAG control	7 ± 1.2
PAG + HIPC (20 μM)	14 ± 0.7
PAG + HIPC (40 μM)	12 ± 0.8

2.5. Cardiac injury assessment and infarct size determination

The measurement of Infarct size (IS) was done by using 1.5% TTC stain in PBS according to previous protocols [12]. The percentage of infarcted tissue (triphenyltetrazolium chloride negative) was measured using Image J software (NIH, USA).

The injury markers mainly lactate dehydrogenase (LDH) and creatine kinase (CK) which are released during cardiac injury were measured in the coronary perfusate and myocardial tissue homogenate as per our previous protocols [2]. The LDH activity was expressed as nmoles of pyruvate liberated/min based on the conversion of lactate to pyruvate. The CK activity was measured from the amount of inorganic phosphate liberated from the conversion of ATP, quantified using ANSA-molybdate reagent.

2.6. Isolation of mitochondrial subpopulation

The differential centrifugation technique was used to isolate rat heart mitochondrial subpopulations, namely interfibrillar mitochondria (IFM) and subsarcolemmal mitochondria (SSM), essentially according to our previous method described elsewhere [13]. IFM and SSM were purified using a 60% percoll gradient and western blot analysis of HSP 60, the marker protein (data not included) against β-actin was used to determine the purity of isolated fractions.

2.7. Measurement of H_2S level and its metabolizing enzymes

2.7.1. Estimation of H_2S in tissue

The H_2S assay was performed according to previously established protocol [14]. Briefly, H_2S trapping agent 1% Zn (O_2CCH_3) was added to cardiac tissue homogenate and centrifuged. The pellet was washed with Milli-Q water twice. To the water resuspended pellet, 40 μL of dye (*N, N*-dimethyl *p*-phenylene diamine in 30 mM $FeCl_3$) was added and incubated for 10 min. The absorbance was read at 670 nm and expressed as μmole of H_2S produced/mg protein using methylene blue as standard.

2.7.2. Cystathionine gamma lyase (CSE) assay

CSE assay was performed according to Stepien and Pieniazer [15].