HYDROGEN SULFIDE PRODUCES CARDIOPROTECTIVE EFFECTS AGAINST ISCHEMIA/REPERFUSION INJURY VIA REGULATION OF INTRACELLUAR PH

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TABLE OF CONTENTS

SUMMARY

Hydrogen sulphide (H_2S) has been identified as the third member of gasotransmitters, alone with nitric oxide (NO) and carbon monoxide (CO). It can be endogenously generated from cysteine by two enzymes, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE). In the current study, the role of hydrogen sulfide (H_2S) in the cardioprotection during ischemia/reperfusion was investigated.

Given that Intracellular pH (pH_i) is an important endogenous modulator of cardiac function and inhibition of Na^+/H^+ exchanger-1 (NHE-1) protects the heart by preventing Ca^{2+} overload during ischemia/reperfusion, the present study investigated the pH regulatory effect of H_2S in rat cardiac myocytes and evaluate its contribution to cardioprotection. It was found that sodium hydrosulfide (NaHS), at a concentration range of 10 to 1000 μM, produced sustained decreases in pH_i in the rat myocytes in a concentration-dependent manner. NaHS also abolished the intracellular alkalinization caused by *trans*-(±) -3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]benzeneacetamide methane-sulfonate hydrate (U50,488H), which activates NHEs. Moreover, when measured with an NH4Cl prepulse method, NaHS was found to significantly suppress NHE-1 activity. Both NaHS and cariporide or [5-(2-methyl-5-fluorophenyl)furan-2-ylcarbonyl]guanidine (KR-32568), two NHE inhibitors, protected the myocytes against ischemia/reperfusion injury. The further functional study showed that perfusion with NaHS significantly improved pos-tischemic contractile function in isolated rat hearts

subjected to ischemia/reperfusion. Blockade of phosphoinositide 3-kinase (PI3K) with 2-(4-morpholinyl)-8-phenyl- 4*H*-1-benzopyran-4-one (LY294002), Akt with Akt VIII, or protein kinase G (PKG) with (9*S*,10*R*,12*R*)-2,3,9,10,11,12-hexahydro-10-methoxy-2,9-dimethyl-1-oxo-9,12-epoxy-1*H*-diindolo[1,2,3-*fg*:3',2',1'-*kl*]pyrrolo[3,4-*i*][1,6]]enzodiazocine-10-carboxylic acid, methyl ester (KT5823) significantly attenuated NaHS-suppressed NHE-1 activity and/or NaHS-induced cardioprotection. Although KT5823 failed to affect NaHS-induced Akt phosphorylation, Akt inhibitor did attenuate NaHS-stimulated PKG activity.

In conclusion, the current work demonstrated that H_2S produced cardioprotection via the regulation of intracellular pH which is achieved by inhibition of NHE-1 activity. Furthermore, this mechanism involves PI3K/Akt/PKG pathway.

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

Symbols	Full name
$[Ca^{2+}]_i$	Intracellular calcium
$[Na^{+}]_i$	Intracellular sodium
2-DOG	2-deoxy-D-glucose
ACE	Angiotensin-converting enzyme
AIF	Apoptosis-inducing factor
ANOVA	One-way analysis of variance
BCECF/ AM	2,7-biscarboxyethyl-5,6-carboxyfluorescein/acetoxymethyl ester
cAMP	Cyclic-adenosine monophospate
CAT	Cysteine aminotransferase
CBE	Cl^-/HCO_3^- exchanger
CBS	Cystathionine beta synthase
CMA	Chaperone-mediated autophagy
CO	Carbon monoxide
COX2	Cyclooxygenase-2
CSE	Cystathionine gamma lyase
Cys	Cysteine
DMEM	Dulbecco's Modified Eagle's Medium

DMSO	Dimethylsulphoxide
GSH	Glutathione
GSSG	Glutathione disulfide
H_2S	Hydrogen sulfide
HOE-642	[4-Isopropyl-3-(methylsulfonyl)benzoyl]guanidine methanesulfonate
I/R	Ischemia/reperfusion
K_{ATP}	ATP-sensitive-Potassium
LVDP	Left ventricular developed pressure
LVeDP	Left ventricular end diastolic pressure
MAPK	p42/44-mitogen activated protein kinase
MetHb	Methhemoglobin
mPTP	Mitochondrial permeability transition pore
MST	Mercaptopyruvate sulfurtransferase
N_2O	Nitrous oxide
$Na_2S_2O_4$	Sodium dithionite
NaHS	Sodium hydrosulfide
NH_4Cl	Ammonium chloride
NHE-1	Na^+/H^+ exchanger
NO	Nitric oxide
OxyHb	Oxyhemoglobin
PGE2	Prostaglandin E2
pH_i	Intracellular pH

PI3K	Phosphoinositol-3-kinase
PKC	Protein kinase C
PKG	Protein kinase G
RAS	Renin–angiotensin system
RSH	Thiol
SO	Sulfite Oxidase
SP	NaHS preconditioning
SulfHb	Sulfhemoglobin
TMB	Tetra-methylbenzidine
TR	Thiosulfate reductase
TS	Thiosulfate sulfurtransferase
TSMT	Thiol S-methyltransferase
+dP/dt	Contractility, maximum gradient during systoles
-dP/dt	Compliance, minimum gradient during diastoles

CHAPTER 1 INTRODUCTION

1.1 Gasotransmitters

1.1.1 Definition of gasotransmitters

The term "gasotransmitter" was firstly introduced in 2002 (Wang 2002). Gasotransmitters, which includes nitric oxide (NO), hydrogen sulphide (H_2S), carbon monoxide (CO), and possibly nitrous oxide (N_2O), is a family of endogenous molecules of gases or gaseous signaling molecules. The following criteria should be met before a gas molecule can be categorized as a gasotransmitter. (i) It is a small molecule of gas; (ii) It is freely permeable to membranes; (iii) It is endogenously and enzymatically generated and its production is regulated; (iv) Its functions have been well and specifically defined at physiologically relevant concentrations; (v) exogenously applying of its counterpart can produce functions of this endogenous molecule; (vi) It should have specific cellular and molecular targets.

1.2 Hydrogen sulfide is the third member of gasotransmitter family

1.2.1 Physical and chemical properties of H_2S

Hydrogen sulphide (H_2S) is a colorless, flammable and naturally occurring gas with a strong rotten egg smell. It is a small molecule soluble in water (1 g in 242 ml at 20°C), organic solvents and lipophilic solvents (Lim, Liu et al. 2008; Li, Hsu et al. 2009). As a weak acid with a pKa of 7.04, H_2S can dissociate in water or plasma as

follows: $H_2S \leftrightarrow HS^- + H^+$ (Wang 2002). H_2S is lipophilic and thus readily permeable and diffusive in the plasma membranes.

1.2.2 Past and current views of H_2S

H_2S was used to be viewed as a toxic gas which is more toxic than hydrogen cyanide (HCN) and CO, and an exposure of H_2S at 300 ppm in air for 30 minutes will result in fatality (Pryor, Houk et al. 2006). Inhibition on cytochrome c oxidase and induction of cell death via formation of reactive oxygen species and mitochondrial depolarization can be the reasons for the toxicity of H_2S. (Dorman, Moulin et al. 2002; Eghbal, Pennefather et al. 2004) Recently, H_2S has been viewed as the third member of gasotransmitters, for the reasons that its concentration in the blood plasma of mice, rats and human is considerably high and its synthesizing enzymes, cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE), are identified.

1.2.3 Biosynthesis of H_2S

H_2S is produced endogenously from cysteine and homocysteine in reactions catalyzed by cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE). These two enzymes are the main players in the metabolism of L-cysteine (Hughes, Bundy et al. 2009) which is the main substrate of the generation of H_2S. The expression of these two enzymes is highly tissue-specific; while CSE is largely expressed in the cardiovascular system, CBS predominates in the central nervous system (Chen, Poddar et al. 1999; Geng, Yang et al. 2004).

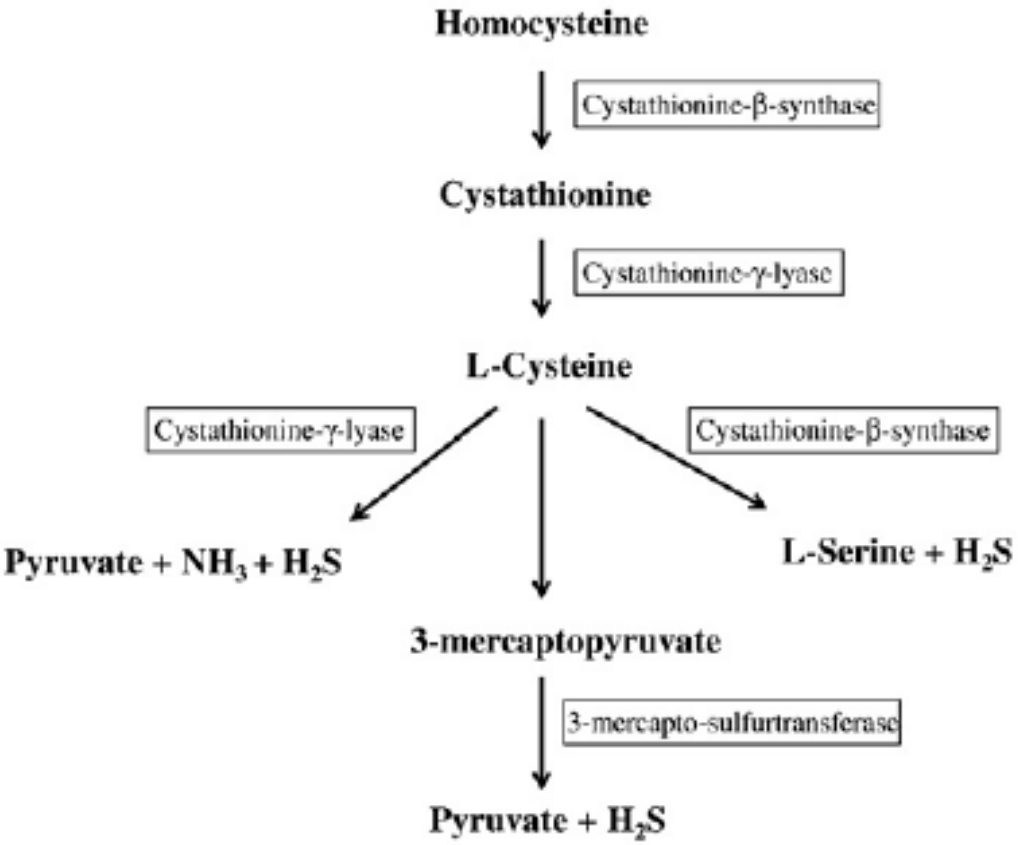

Figure 1 Three pathways of endogenous synthesis of H_2S.

This figure is taken from Hughes (2009).(Hughes, Bundy et al. 2009)

1.2.4 Metabolism of endogenous H_2S

Oxidation in mitochondria, methylation in cytosol and scavenging by methemoglobin or metallo- or disulfide-containing molecules are three major pathways in H_2S metabolism (Wang 2002). Briefly, H_2S is metabolized in mitochondria initially to thiosulphate which is further converted to sulfate which is the end-product and is eventually excreted by the kidney (Beauchamp, Bus et al. 1984; Lowicka and Beltowski 2007). Also, H_2S could be methylated in the cytosol by thiol S-methyltransferase (TSMT) and be turned into methanethiol and dimethylsulfide(Furne, Springfield et al. 2001). Finally, H_2S could be scavenged by methemoglobin to form sulfhemoglobin (Lowicka and Beltowski 2007).

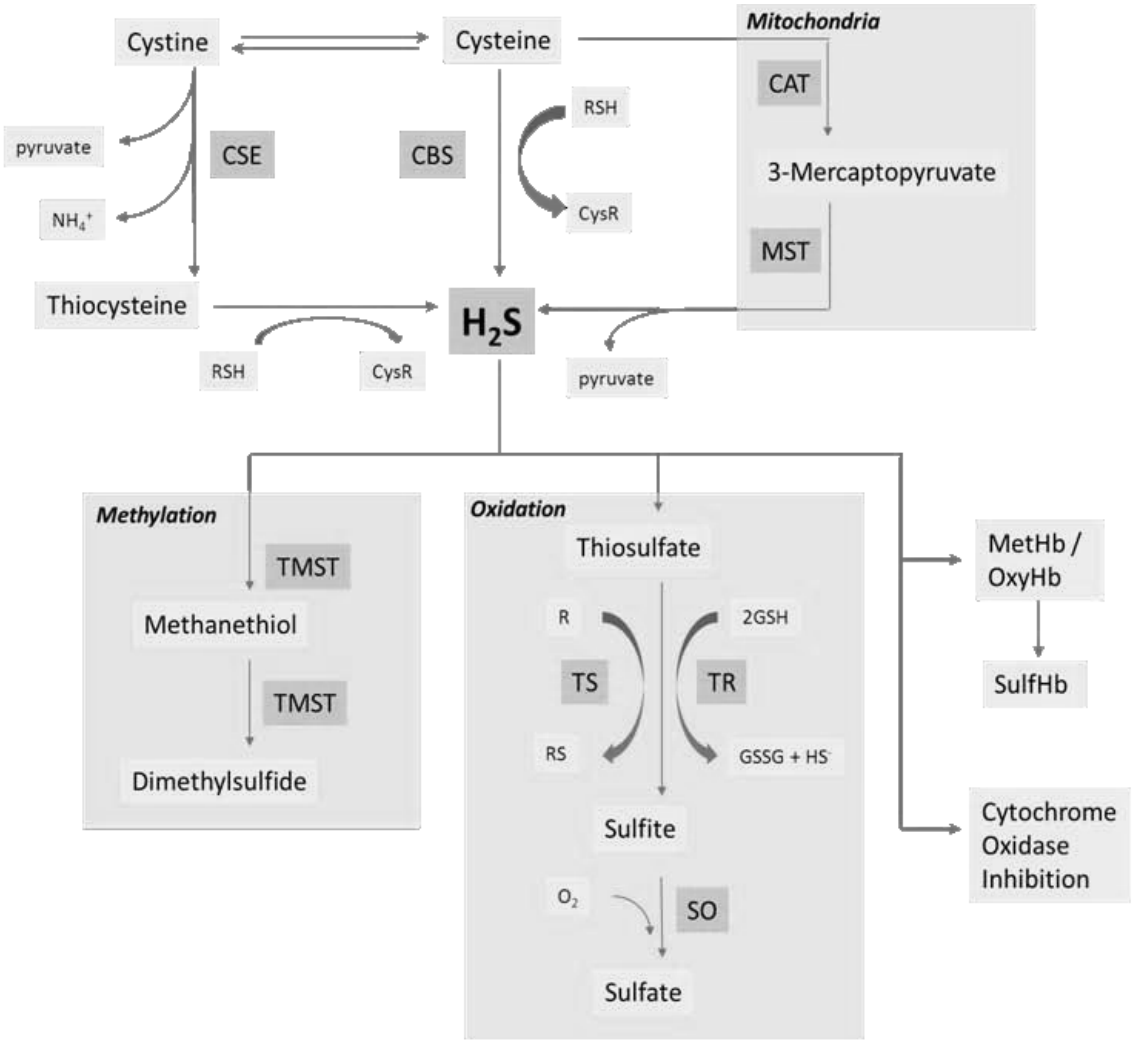

Figure 2 Endogenous H_2S synthesis and metabolism.

CSE: Cystathionine gamma lyase; CBS: Cystathionine beta synthase; MST: Mercaptopyruvate sulfu rtransferase; CAT: Cysteine aminotransferase; TR: Thiosulfate reductase; TS: Thiosulfate sulfurtransferase; SO: Sulfite Oxidase; GSH: Glutathione; GSSG: Glutathione disulfide; RSH: Thiol Cys: Cysteine MetHb: Methhemoglobin OxyHb: Oxyhemoglobin SulfHb: Sulfhemoglobin (Ang 2011)

1.3 Physiological functions of H_2S in the cardiovascular system

1.3.1 Vasorelaxant effects of H_2S

H_2S showed its vasorelaxant effect through activating K_{ATP} channel in thoracic aorta (Zhao, Zhang et al. 2001). This effect was also observed in mesenteric arteries (Cheng, Ndisang et al. 2004), portal vein (Hosoki, Matsuki et al. 1997) and ileum (Teague, Asiedu et al. 2002). Moreover, H_2S could decrease bold pressure when a bolus was injected into rats (Zhao, Zhang et al. 2001; Ali, Fazl et al. 2006).

1.3.2 Physiological functions of H_2S in the cardiovascular system

H_2S plays an important role in the regulation of heart function. Both endogenous and exogenous H_2S protects heart from isoproterenol-induced myocardial injury by directly scavenging oxygen free radicals (Geng, Chang et al. 2004) and inhibiting the adenylyl cyclase/cAMP pathway or L-type calcium channel (Yong, Pan et al. 2008). Till now, plenty of studies have demonstrated that H_2S could protect the heart from myocardial injury (Johansen, Ytrehus et al. 2006; Sivarajah, McDonald et al. 2006; Zhu, Wang et al. 2007). Moreover, H_2S preconditioning (SP) mimicked cardiac protective effects produced by ischemic preconditioning (Pan, Feng et al. 2006). Although it is generally accepted that H_2S could produce cardioprotective effects in the hearts subjected to ischemia injury, the exact mechanism has yet remained unclear.

1.4 Signaling Mechanisms of H_2S

1.4.1 Activation of K_{ATP} channels

An ATP-sensitive potassium channel (K_{ATP} channel) is a type of potassium channel that is gated by ATP and composed of two kinds of subunits: the pore forming subunits, inwardly rectifying potassium channel subunits (KIR6.1 or KIR6.2), and the larger regulatory subunits, sulphonylurea receptor (SUR). They can be further identified by their position within the cell as the sarcolemmal K_{ATP} channel, mitochondrial K_{ATP} channel, and nuclear K_{ATP} channel (Zhuo, Huang et al. 2005).

K_{ATP} channel is involved in metabolite regulation. In cardiomyocytes, energy is derived mostly from long-chain fatty acids and their acyl-CoA equivalents. During ischemia reperfusion, the oxidation of fatty acids slows down, which results in the accumulation of acyl-CoA and K_{ATP} channel opening (Koster, Knopp et al. 2001).

More importantly, many studies have demonstrated that the effect of H_2S in cardiovascular system is related to the opening of K_{ATP} channels, such as the vasodilatory effect of H_2S (Zhao, Zhang et al. 2001), the protective effect of H_2S in cardiac myocytes (Bian, Yong et al. 2006; Sivarajah, McDonald et al. 2006), and the negative effect of H_2S on myocardial contractility (Geng, Yang et al. 2004)

1.4.2 Stimulation of MAP Kinases

Mitogen-activated protein (MAP) kinases are serine/threonine-specific protein kinases. MAPKs are involved in directing cellular responses to various stimuli and

regulate proliferation, cell survival, and apoptosis (Pearson, Robinson et al. 2001). The first-discovered MAPK was ERK1 (MAPK3). ERK1 and the closely related ERK2 (MAPK1) are both involved in growth factor signaling. As regulators of cell proliferation, they have a highly specialized function. Also, c-Jun N-terminal kinases (JNKs), and p38 MAPKs have been well characterized in mammals. Both JNK and p38 signaling pathways are responsive to stress stimuli, such as ultraviolet irradiation and heat shock, and are involved in cell apoptosis.

Interestingly, studies have shown that ERK1/2 is one of the downstream target for H_2S in HEK293 cells (Yang, Cao et al. 2004), human aorta smooth muscle cells (Yang, Sun et al. 2004; Yang, Wu et al. 2006), human monocytes (Zhi, Ang et al. 2007), and in cardiomyocytes (Hu, Chen et al. 2008). Although one of our studies has suggested the involvement of p38 MAP kinase in anti-inflammatory role of H_2S (Hu, Wong et al. 2007), this conclusion is not widely accepted.

1.4.3 Other signaling mechanisms of H_2S

Moreover, researchers have recently found that pre- and post-conditioning with H_2S produced cardioprotective effects against ischemic injury via regulation of protein kinase C (PKC), cyclooxygenase-2 (COX-2), NO, phosphoinositol-3-kinase (PI3K)/Akt and GSK3β pathways (Bian, Yong et al. 2006; Hu, Pan et al. 2008; Yong, Lee et al. 2008; Yao, Huang et al. 2010). More importantly, endogenous H_2S was found to contribute to the cardioprotection induced by ischemic pre- and post-conditioning (Bian, Yong et al. 2006; Pan, Feng et al. 2006; Yong, Lee et al. 2008). In addition, H_2S

may also produce a pro-angiogenic effect (Cai, Wang et al. 2007), which can contribute to its cardioprotective action. These results suggest that H_2S not only ameliorates the pathological process of ischemic heart disease but may also act as a cardioprotective regulator.

1.5 H_2S under pathological condition

During pathological process, a change of H_2S level has been reported in different animal models. In the cardiovascular system, this change is usually relevant to CSE activity. Scientists found that H_2S concentration decreased significantly in patients with coronary heart disease (Jiang, Wu et al. 2005), in myocardial tissue subjected to myocardio injury (Geng, Chang et al. 2004) and in medium of isolated cardiomyocytes treated with ischemia solution (Bian, Yong et al. 2006). On the other side, elevation of H_2S level was also observed by different groups in a LPS-injection septic shock mice model (Li, Bhatia et al. 2005), in endotoxemia rat model (Collin, Anuar et al. 2005) and in the liver and pancreas in Streptozotocin-induced diabetic rats (Yusuf, Kwong Huat et al. 2005).

1.6 intracellular pH and ion exchangers

Intracellular pH (pH_i) is an important modulator of cardiac function, influencing processes as varied as contraction, excitation and electrical rhythm. Regulation of pH_i is required for the maintenance of an environment appropriate for cellular activities. Hence, pH_i has to be tightly controlled within a narrow range, largely through the

activity of transporters such as Na^+/H^+ exchanger (NHE-1) and Cl^-/HCO_3^- exchanger (CBE). Protons are produced metabolically within the heart. These ions are highly reactive with cellular proteins and they must be removed if cardiac function is to be maintained. During ischemia, lactic acid accumulation causes significant intracellular acidosis, which stimulates NHE-1. This minimizes the intracellular acidosis and causes an increase in intracellular sodium ($[Na^+]_i$). The protons leaving the cell accumulate produce an extracellular acidosis. During reperfusion, the extracellular protons are flushed away and the activity of NHE-1 would then lead to a rapid recovery of pH_i and a rise in $[Na^+]_i$. The latter could eventually result in Ca^{2+} entry by means of Na^+/Ca^{2+} exchangers. Therefore, it is well accepted that inhibition of NHE-1 protects against some of the damaging effects of ischemia. We recently reported that H_2S regulates pH_i in vascular smooth muscle cells (Lee, Cheng et al. 2007) and glial cells (Lu, Choo et al. 2010).

Cellular organelles and compartments	pH
Cytosol	7.2
Nucleus	7.2
Endoplasmic reticulum	7.2
Golgi network	6-6.7
The matrix of mitochondrial	8
Peroxisomes	7
Lysosome	4.7

Table 1 The pH values of individual cellular organelles and compartments in a prototypical mammalian cell
(Casey, Grinstein et al. 2010)

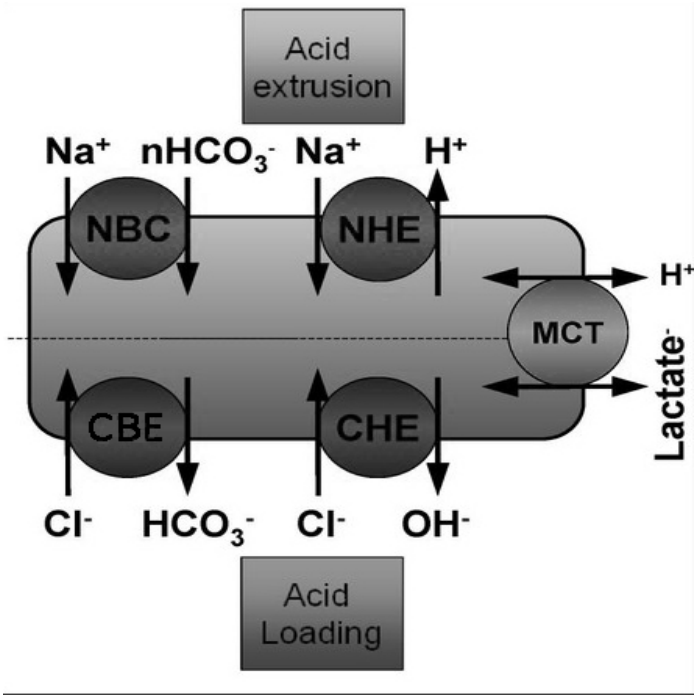

Figure 3 Ion exchangers regulate intracellular pH

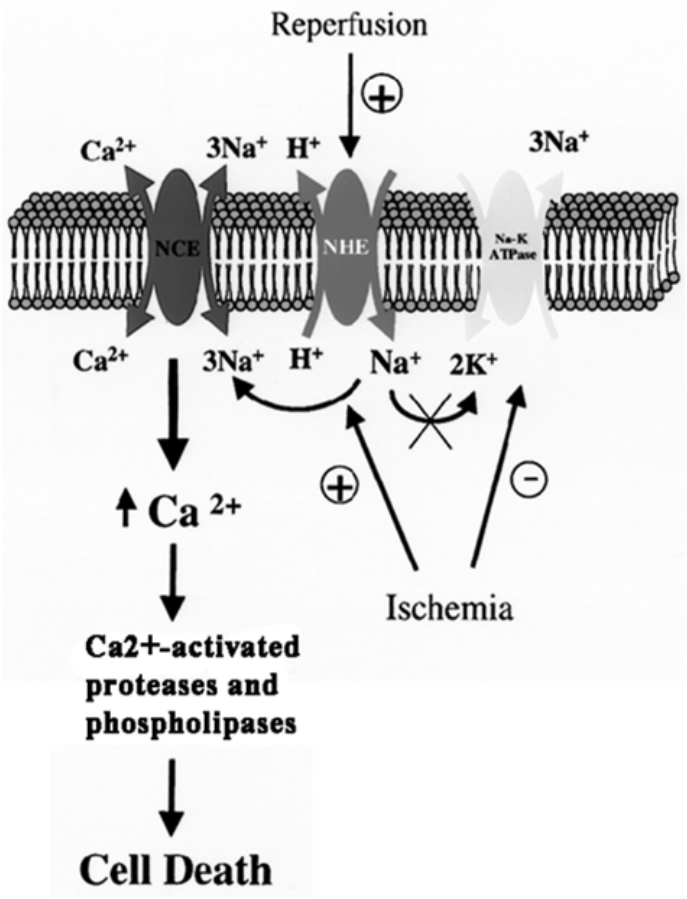

Figure 4 Cell death induced by ischemia/reperfusion via regulation of ion exchangers

1.7 Hypotheses and Objectives

Contributions from scientists studying H_2S effects on cardioprotection help us to unveil the physiological roles of H_2S. Until now, most of the studies of downstream target of H_2S have focused on protein kinase C (PKC), K_{ATP} channels, cyclooxygenase-2 (COX-2), p42/44-mitogen activated protein kinase (MAPK) and phosphoinositol-3-kinase (PI3K)/Akt pathways. However, whether or not H_2S can also produce cardioprotective effect via regulation of pH_i in hearts by affecting NHE-1 activity is still unknown. Thus, my project was therefore designed to determine whether the mechanisms of cardioprotection produced by H_2S against ischemia/reperfusion involve the effect of H_2S on pH_i in isolated cardiac myocytes and rat hearts, and to investigate whether this mechanism involves the activation of PI3K/Akt pathway and/or protein kinase G (PKG).

CHAPTER 2 MATERIALS and METHODS

2.1 Isolation of rat ventricular cardiac myocytes

The study protocol was approved by the Institutional Animal Care and Use Committees (IACUC) of National University of Singapore. Sprague-Dawely rats (190~210 g, male) were anesthetized with intraperitoneal (*i.p.*) injection of a combination of ketamine (75mg/kg) and xylazine (10mg/kg). Heparin (1000 IU) was administered *i.p.* to prevent coagulation during removal of the heart. The rat hearts was quickly excised, mounted on a Langendorff apparatus, and perfused in a retrograde fashion via the aorta with calcium-free Tyrode's solution (in mmol/L: 137 NaCl, 5.4 KCl, 1 $MgCl_2$, 10 HEPES, 10 Glucose, pH 7.4) at 37 °C. After 5 min the perfusion solution was changed to the Tyrode's solution containing 1 mg/ml collagenase type I and 0.28 mg/ml protease (type XIV) and perfused for a further 25-30 min. The perfusion solution was then changed to Ca^{2+}-Tyrodes solution containing 2×10^{-4}mol/L $CaCl_2$ without enzymes for an additional 5 min. The ventricular tissue was then cut into small pieces in a Petri dish containing pre-warmed Ca^{2+} Tyrode's solution and shaken gently to ensure adequate dispersion of dissociated cardiac myocytes. A 2.5×10^{-4} meter mesh screen was used to separate the isolated cardiac myocytes from cardiac tissue. The cells were then washed three times in Ca^{2+}-Tyrode's solution and collected by centrifugation (700 rpm, for 1 min). Ca^{2+} concentration of the Tyrode's solution was increased gradually to 1.25×10^{-3} mol/L in 20 min. More than 80% of the cells were

rod-shaped and impermeable to trypan-blue. The cells were allowed to stabilize for 30 min before any experiments.

2.2 pH_i measurements in rat ventricular cardiac myocytes

The isolated cardiac myocytes were incubated with 1 μM 2,7-biscarboxyethyl-5,6-carboxyfluorescein/acetoxymethyl ester (BCECF/ AM) for 30 min in the dark at room temperature. The unincorporated dye was then removed by washing the cardiac myocytes twice in fresh incubation solution. The membrane-permeable ester was trapped inside the myocytes because of hydrolyzation by esterases and fluoresced pH-dependently. The loaded rat cardiac myocytes were kept in the dark at room temperature for another 30 min before pH_i measurement to allow the BCECF/AM in the cytosol to de-esterify.

The BCECF/AM-loaded rat cardiac myocytes were transferred to the stage of an inverted microscope (Nikon, Tokyo, Japan) in a perfusion chamber at room temperature. The inverted microscope was coupled with a dual-wavelength excitation spectrofluorometer (Intracellular Imaging Inc., Cincinnati, OH). Cells were perfused with Krebs' solution (117 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1.25 mM $CaCl_2$, 25 mM $NaHCO_3$, and 11 mM glucose, pH 7.4). Drugs were then added directly into the bath solution during pH_i measurements, and the change in fluorescent intensity was monitored. The pH-dependent fluorescent signal of BCECF/AM was obtained by illuminating at excitation wavelengths of 490 nm (F490) and 440 nm (F440). The ratio of signals obtained at F490 and F440 was used to represent pH_i. The

calibration of BCECF/AM signals was performed by setting pH_i to extracellular pH with 10 μM nigericin in Krebs' solution. The extracellular pH was changed by perfusion with Krebs' solution at pH 6.8, 7.4, 8, or 10. From these corresponding pH and fluorescence measurements, a graph was constructed and used for the translation of fluorescence values into pH_i values.

2.3 Determination of NHE-1 activity

NHE-1 activity in cardiac myocytes was assessed by measuring the recovery rate of cells from intracellular acidification. Cells were perfused with HEPES-buffered solution containing 137 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose, adjusted to pH 7.4 with NaOH. Myocytes were maintained at 25°C throughout and subjected to intracellular acidosis by transient exposure to 20 mM NH_4Cl (6 min at 25°C) with subsequent washout for 8 min. Because $NH4^+$ enters the cell at a slow, but significant, rate on transporter pathways, this feature is used to acid-load the cytoplasm (Fro¨hlich and Wallert, 1995). Washout of NH_4Cl therefore imposes an acid load by trapping protons into the myocytes. Subsequently, myocytes recovered from this acid load via the activity of NHE-1. The slope of pH_i recovery determines the sarcolemmal NHE-1 activity (Fro¨hlich and Wallert, 1995). To assess NHE-1 activity of myocytes in the presence of H_2S, 0.1 mM sodium hydrosulfide (NaHS) was added to the cells 10 min before intracellular alkalinization, followed by perfusion with NaHS-containing HEPES-buffered solution.

2.4 Determination of CBE activity

CBE activity was assessed by measuring the recovery rate of cells from intracellular alkalinization. Intracellular alkalinization was introduced by a rapid addition of 20 mM NH_4Cl to bathing solution. Exposure of cells to NH_4Cl results in diffusion of NH_3 across cell membranes, leading to rapid intracellular alkalinization (Furtado 1987). pH_i gradually decreases from the peak of alkalinization due to efflux of HCO_3^- via activity of CBE, that is, a recovery from alkali load (Xu and Spitzer 1994). The slope of the pH_i decrease determines the rate of recovery from the peak of alkalinization and is measured in ΔpH/msec. To assess the CBE activity of cells in the presence of H_2S, 0.1 mM NaHS was added 10 min prior to intracellular alkalinization and myocytes were perfused with NaHS-containing HEPES-buffered solution.

2.5 Ischemia/reperfusion in isolated rat ventricular myocytes

For the cardiac myocytes ischemia/reperfusion experiments, simulated ischemia solution (*i.e.* glucose-free Krebs buffer containing 10 mM 2-deoxy-D-glucose (2-DOG), an inhibitor of glycolysis and 10 mM sodium dithionite ($Na_2S_2O_4$), an oxygen scavenger, pH 6.6) was applied. The use of simulated ischemia solution in this way produces a mixture of effects including metabolic inhibition, anoxia and acidosis. This method was adapted from previous publications (Ho, Wu et al. 2002). In brief, after dissociation, the cardiac ventricular myocytes were allowed to stabilize for 30 min before the experiment commenced. Ventricular myocytes were subjected to ischemia solution for 30 min followed by reperfusion with Dulbecco's Modified

Eagle's Medium (DMEM) solution for up to one hour. NaHS or cariporide was applied 10 min before and during ischemia, respectively. Myocyte viability was determined at the end of reperfusion for a certain period as specified in the individual results.

2.6 Cell viability assay for rat ventricular cardiac myocytes

Trypan blue exclusion was used as an index of myocyte viability. At the end of reperfusion, cardiac myocytes were incubated with 0.4% (w/v) trypan blue dye (Sigma) for 3 min. Those unstained were termed to be non-blue cells. The ratio of non-blue cells/total cells was determined in a hemocytometer chamber under a light microscope.

2.7 PKG activity assay

Cyclic GMP-dependent protein kinase assay (Cyclex, MBL International Corporation) was used to measure PKG activity. The isolated myocytes were divided into different treatment groups: vehicle + ischemia group (myocytes treated with vehicle and subjected to simulated ischemia solution for 30 min), NaHS + ischemia group (myocytes were pretreated with NaHS at 0.1 mM for 10 min before subjected to ischemia), Akt VIII + NaHS + ischemia (myocytes were treated with Akt VIII at 1 μM for 10 min and then NaHS at 0.1 mM for 10 min followed by ischemia for 30 min), and KR + NaHS + ischemia (myocytes were treated with KR-32568 at 1 μM for 10 min and then NaHS at 0.1 mM for 10 min followed by ischemia for 30 min).

Protein extraction was performed, and 100μl of each sample was transferred into a 96-well plate pre-coated with a substrate corresponding to recombinant G-kinase substrate and incubated at 30℃ for 30 min. During the incubation, the substrate was phosphorylated by PKG in the protein sample, which was measured by incubating the substrate with a horseradish peroxidase conjugate of 10H11, a anti-phospho-G-kinase substrate threonine 68/119 specific antibody, for 1 hour at room temperature. Then the chromogenic substrate tetra-methylbenzidine (TMB) was added into the wells by adding the substrate reagent, which converted the colorless solution to a blue solution. After the stop solution was added into the wells, Absorbance was determined at 450 nm using a 96 well microplate reader (Tecan Systems Inc., U.S.A.). Experiments of "Test Sample cGMP minus" group and "ATP minus" group were conducted as quality controls of our assay.

2.8 Western blotting analysis

To examine the effect of NaHS on non-ischemic myocytes, the isolated cardiac myocytes were subjected to NaHS or vehicle treatment for 30 min. To test the action of NaHS on cardiac myocytes subjected to ischemic insults, NaHS (0.1 mM) was added to the myocytes for 10 min before and during ischemia for 30 min. To examine the regulatory effect of PKG on Akt phosphorylation, KT5823 (0.5 μM), a specific inhibitor of PKG, was added 10 min before NaHS treatment. At the end of treatment or ischemia, myocytes were gently washed twice with ice-cold PBS, homogenized in chilled lysis buffer containing 125 mM NaCl, 25 mM Tris (pH 7.5), 5 mM EDTA, 1%

NP-40, 1 mM NaF, 2 mM Na_3VO_4 and protease inhibitor (Roche) and then shaken on ice for one hour. After that, the lysates were centrifuged at 13,000 ×g for 10 min at 4°C. The supernatants were then collected and denatured by SDS sample buffer, and the epitopes were exposed by boiling the protein samples for 5 min in a dry heat block. Equal amount of proteins were loaded and separated on 12% SDS-PAGE gel, and then transferred onto nitrocellulose membrane. The membrane was then probed with antibodies against phosphorylated- and total Akt (Cell Signaling, 1:1000) and second antibody(Santa Cruz Biotechnology, CA, USA). Immunoreactivity was detected using an ECL Western blot detection kit (Amersham Biosciences, USA).

2.9 Langendorff heart preparation and haemodynamic assessment

Hearts were quickly excised, mounted on a Langendorff apparatus, and perfused in a retrograde fashion via the aorta with Kreb's buffer (137 mM NaCl, 2.5 mM $CaCl_2$, 5 mM KCl, 1.2 mM $MgSO_4$, 10 mM HEPES, and 10 mM glucose, pH 7.4) at 37^{o}C. The hearts were perfused at a constant pressure of 80 mmHg. These were subsequently submitted to 30 min stabilization, 30 min global no-flow ischemia and 1 h reperfusion. NaHS in Kreb's solution was perfused into heart for 10 min before the global no-flow ischemia was commenced. During no-flow ischemic period, no solution was perfused. Hearts was continued to be exposed to the solution containing NaHS during the no-flow ischemia period.

Left ventricular pressure was monitored using a latex balloon connected to a pressure transducer (Powerlab, Australia). The balloon volume was adjusted to obtain a

left ventricular end-diastolic pressure (LVeDP) of 5–8 mmHg. All data were digitally converted and stored on a computer for analysis (Powerlab, Australia).

2.10 Chemicals and reagent

Nigericin, U50,488H, NH_4Cl, Sodium hydrosulfide (NaHS), KR-32568, collagenase I, protease XIV and Trypan blue dye (0.4%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2,7-biscarboxyethyl-5,6-carboxyfluorescein/AM (BCECF/AM) were obtained from Molecular Probes (Eugene, OR, USA). KT5823, Akt VIII and LY294002 were obtained from Merck (Nottingham, UK). Nigericin is dissolved in ethanol. All other chemicals were dissolved in water except KR-32568, KT5823, LY294002, BCECF/AM and DIDS, which were dissolved in dimethylsulphoxide (DMSO).

Cariporide ([4-Isopropyl-3-(methylsulfonyl)benzoyl]guanidine methanesulfonate, HOE-642) was a gift from Sanofi Aventis, Germany. In our preliminary study, we tested the dose-dependent effect of cariporide at doses of 1, 5, 7 μM on cell viability and NHE activity in the isolated rat cardiac myocytes and found that cariporide produced significant effects only when at a concentration of 7 μM. Therefore, this concentration was used in all further experiments.

NaHS was used as H_2S donors. The use of NaHS enables us to define the concentrations of H_2S in solution more accurately than bubbling H_2S gas. NaHS at concentrations used in our work did not change the pH of the medium (Geng, Yang et al. 2004).

2.11 Statistical analysis

Values presented are Mean ± SEM. Paired Student's *t*-test was used to determine the difference in fluorescent signal before and after treatment in the same cell. One-way analysis of variance (ANOVA) was used with a post-hoc (Bonferroni) test to determine the differences among groups. The significance level was set at $P<0.05$.

CHAPTER 3 RESULTS

3.1 Cardioprotection induced by hydrogen sulfide in rat hearts and rat cardiac myocytes

3.1.1 NaHS produced protective effect on hemodynamic function in isolated hearts

Hemodynamic disorders always lead to the disturbances in the blood movement and the interference in transfer of O_2 and glucose into the cells. To examined the effect of NaHS on contractile function of isolated heart during ischemic reperfusion, I perfumed hemodynamic assessment. Figure 5 shows the representative tracings of the effect of NaHS (100 μM) on isolated hearts subjected to ischemia/reperfusion. Hearts of control group could not function normally, however NaHS-treated hearts restored beating after several minutes of reperfusion.

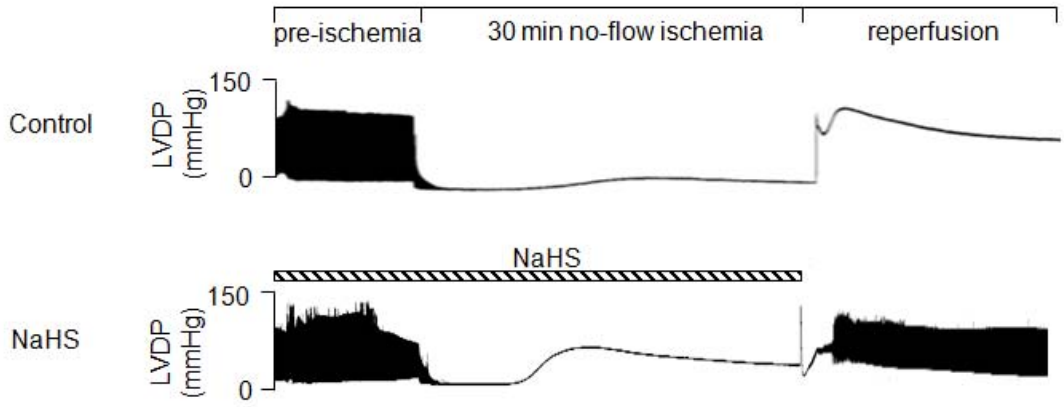

Figure 5 Representative tracings of left ventricular developed pressure (LVDP) of control and NaHS (100 μM) treatment group.

During the whole period of ischemia/reperfusion, all parameters of hemodynamics were recorded and some of them were chosen for analysis.

Left ventricular developed pressure (LVDP) was calculated as the difference between left ventricular systolic pressure and left ventricular diastolic pressure. As shown in Figure 6, LVDP of NaHS group increased to 47.81% ± 5.59% of preischemia value at the end of 15 min reperfusion, while LVDP of control group didn't recovered at all and was kept at 7.67% ± 3.19% of preischemia value, which indicates that NaHS treatment effectively restored LVDP during the reperfusion period.

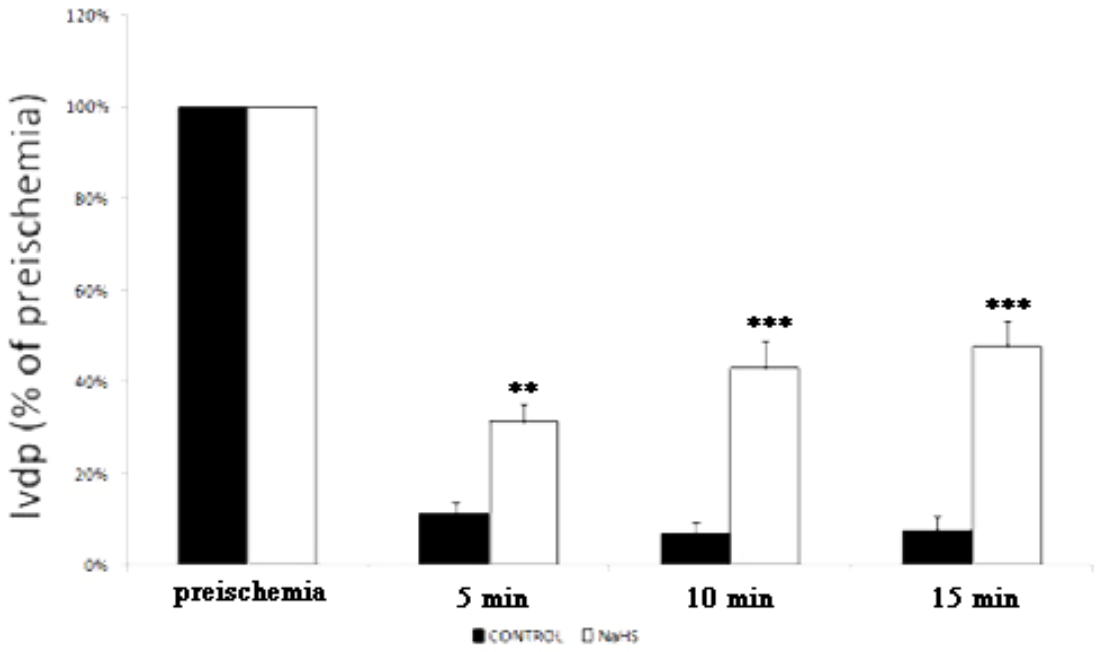

Figure 6 The cardioprotective effect of H_2S on left ventricular developed pressure (LVDP).

Pretreatment with NaHS (100μM) for 10 min significantly attenuated the effects of ischemia/reperfusion on LVDP. Mean±S.E.M. (n = 6–8). *, p<0.05; **, p<0.01; ***, p<0.001 versus the corresponding values in the control group.

Figure 7 demonstrates the significant difference of left ventricular end diastolic pressure (LVeDP), the minimum pressure recorded during diastoles, between control group and NaHS group. LVeDP of NaHS group slowly decreased to 762.92% ±148.21% of the value before ischemia, while LVeDP of control group at 15 min of reperfusion remained at 1273.32% ± 232.00% of the value before ischemia. Our data suggest that hearts of control goup could not totally relax during reperfusion.

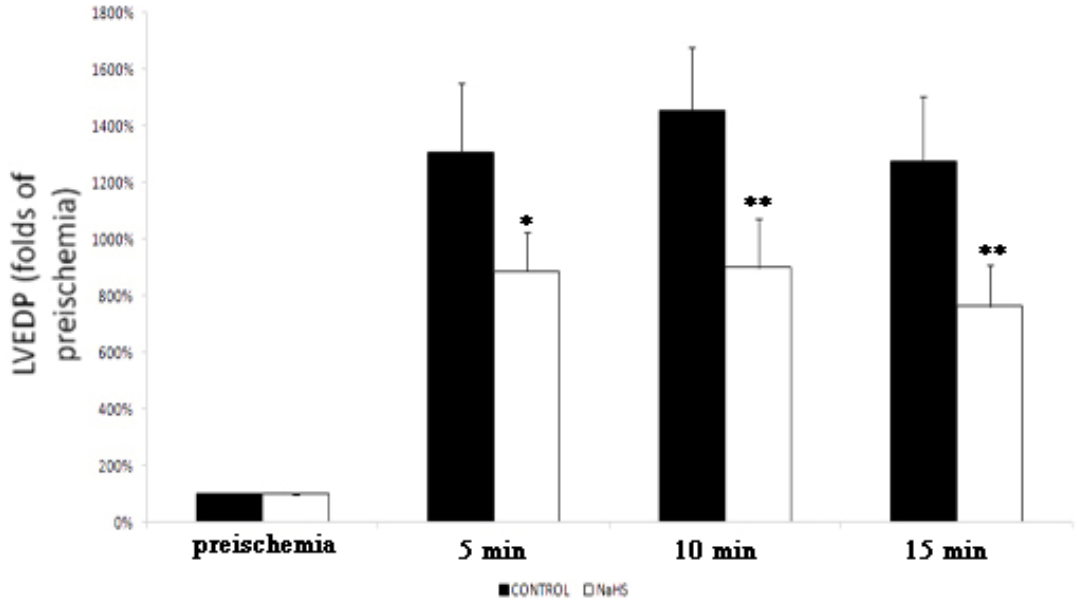

Figure 7 The cardioprotective effect of H_2S on left ventricular end diastolic pressure (LVeDP).

Pretreatment with NaHS (100μM) for 10 min significantly attenuated the effects of ischemia/reperfusion on LVeDP. Mean±S.E.M. (n = 6–8). *, p<0.05; **, p<0.01; ***, p<0.001 versus the corresponding values in the control group.

Contractility (+dP/dt) was represented by the maximum gradient during systoles and compliance (-dP/dt) was represented by the minimum gradient during diastoles. Figure 8&9 show that pretreatment with NaHS restored contractility and compliance of rat hearts to 46.20% ±7.23% and 79.79% ±12.07% respectively, when compared with the preischemia value. On the other hand, the recovery of +dP/dt and -dP/dt of control group was limited to only 5.35% ± 2.31% and 7.02% ± 3.03% of preischemia value at the first 15 min of reperfusion. These data indicate that NaHS treatment significantly restored the contractility and compliance of hearts subjected to ischemia/reperfusion injury.

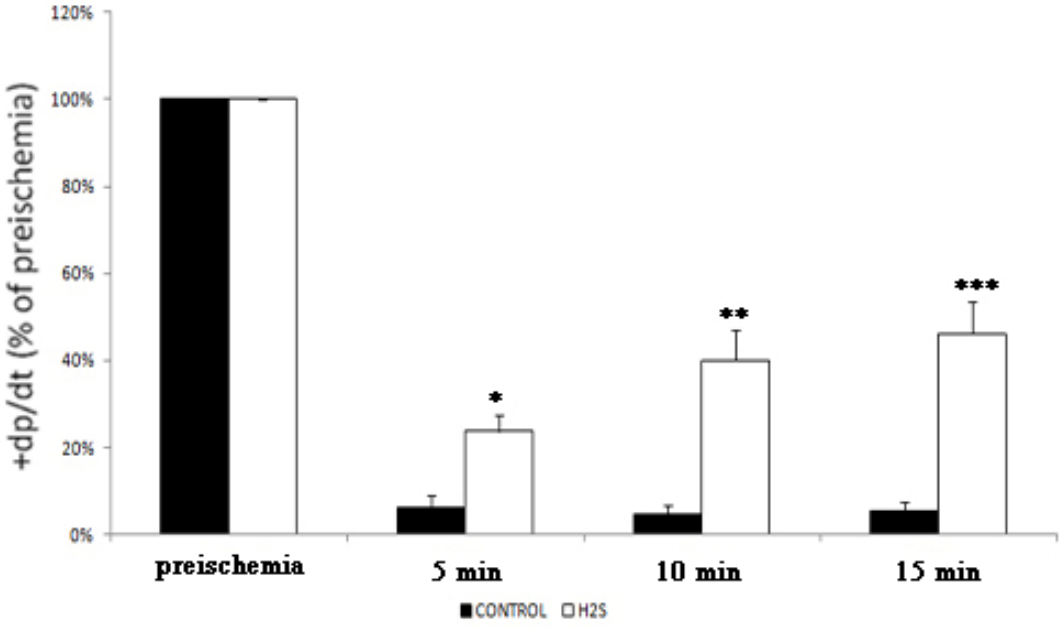

Figure 8 The cardioprotective effect of H_2S on maximum gradient during systoles (+dP/dt).

Pretreatment with NaHS (100μM) for 10 min significantly attenuated the effects of ischemia/reperfusion on +dP/dt. Mean±S.E.M. (n = 6–8). *, p<0.05; **, p<0.01; ***, p<0.001 versus the corresponding values in the control group.

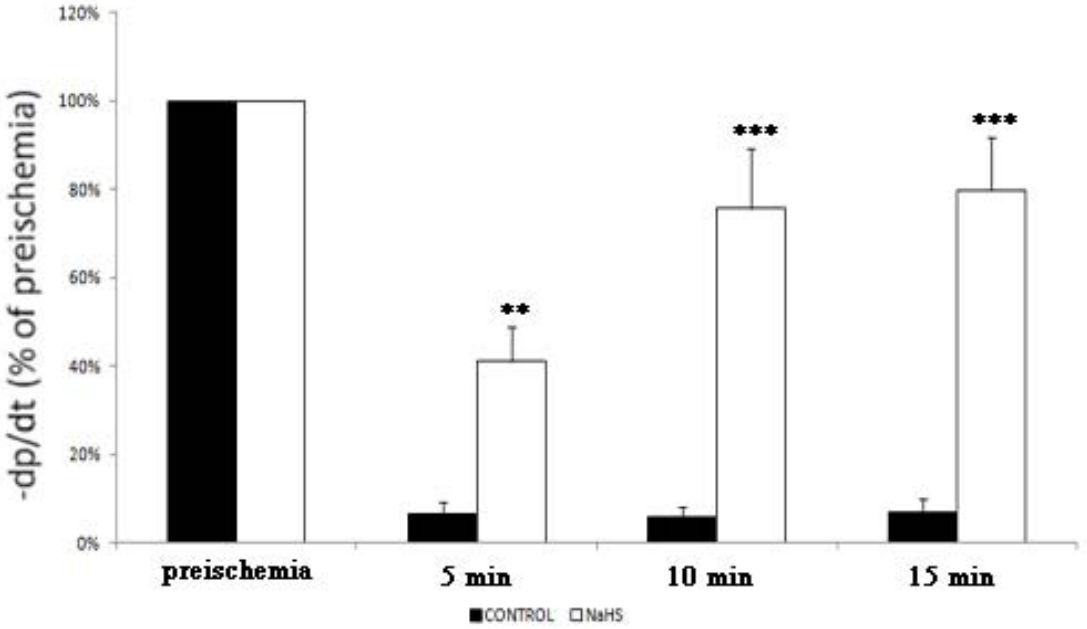

Figure 9 The cardioprotective effect of H_2S on minimum gradient during diastoles (-dP/dt).

Pretreatment with NaHS (100μM) for 10 min significantly attenuated the effects of ischemia/reperfusion on -dP/dt. Mean±S.E.M. (n = 6–8). *, p<0.05; **, p<0.01; ***, p<0.001 versus the corresponding values in the control group.

Taken together, these data suggest that H_2S elicited significant cardioprotective effect on hemodynamic function represented by LVDP, LVeDP, +dP/dt, and -dP/dt at the first 15 min of reperfusion.

3.1.2 Effects of NaHS on cell viability in rat cardiac myocytes subjected to ischemia/reperfusion insults

Since NHE-1 inhibition may protect hearts against ischemia/reperfusion-induced injury (Karmazyn 2001), we further tested whether NaHS could also protect the cardiac myocytes against ischemia/reperfusion injury. The experimental protocol is shown in Figure 10A and described in the section on Materials & Methods. As shown in Figure 10B, both NaHS and cariporide significantly increased cell viability in ventricular myocytes subjected to ischemia (30 min)/reperfusion (10 min), suggesting that like the NHE-1 inhibitor, NaHS may also protect cardiac myocytes against ischemia/reperfusion-induced injury. We further examined the time-course for the cardioprotection offered by NaHS. As shown in Figure 10C, NaHS (100 μM) treatment protected cardiac myocytes subjected to ischemia (30 min)/reperfusion for up to 30 and 60 min. These data suggest that the cardioprotective effects of NaHS may last at least for 1 h after reperfusion.

To examine whether the effect of NaHS was additive to that caused by NHE-1 inhibition, we compared the cardioprotective effects conferred by NaHS, KR-32568 (1μM, a potent NHE-1 inhibitor) alone or in combination (NaHS+KR-32568). As shown in Figure 10D, NaHS produced comparable protective effects to those caused by KR-32568 alone and the combination treatment. These data suggest that H_2S and the NHE-1 inhibitor did not produce synergistic effects.

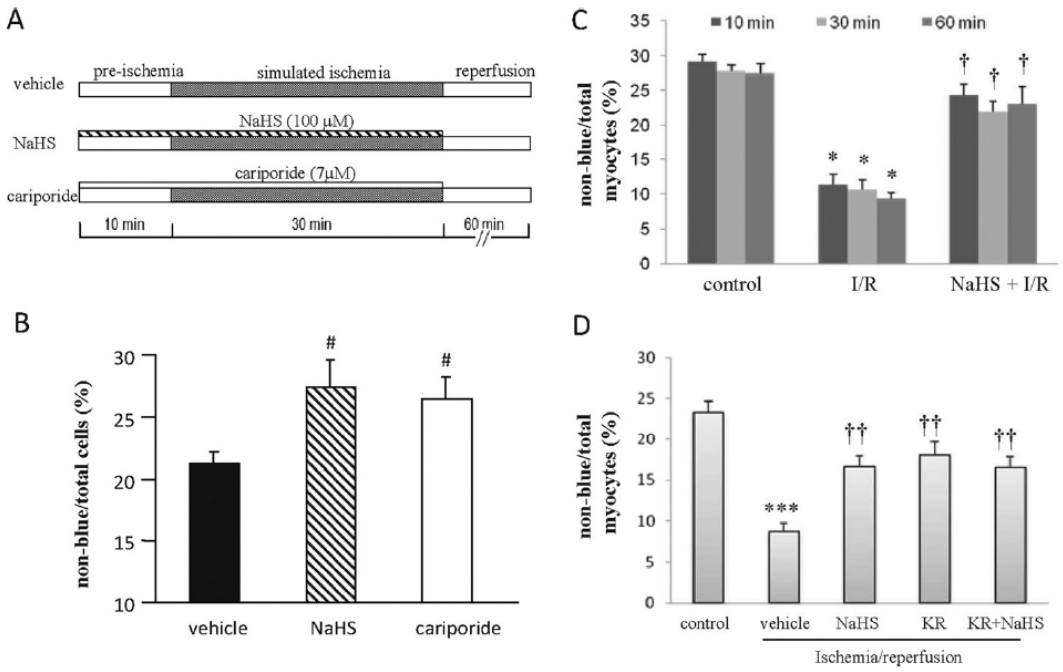

Figure 10 Effect of NaHS on cell viability in cardiac myocytes subjected to ischemia/reperfusion (I/R).

A, experimental protocol. The cardiac myocytes were treated with vehicle, NaHS (100 μM), or cariporide (7 μM) for 10 min before and during the simulated ischemia. After ischemia for 30 min, the simulated ischemia buffer was washed out with fresh Dulbecco's modified Eagle's medium solution, and cell viability was measured at the end of reperfusion with a Trypan blue exclusion assay. B, both NaHS and cariporide significantly increased the cell viability of cardiac myocytes subjected to ischemia (30 min)/reperfusion (10 min). C, group data show the protective effects of NaHS against the cardiac myocytes injury induced by ischemia and reperfusion for 10, 30, and 60 min. D, treatment with NaHS (100 μM) and KR (KR-32568; 1 μM) alone or a combination of both protected myocytes against ischemia/reperfusion insult to a similar extent. Mean±S.E.M. (n=4–6). #, P<0.05 versus the vehicle-treated group. *, p<0.05; ***, p<0.001 versus the corresponding value in the control group. †, P<0.05; ††, P<0.01 versus the corresponding value in the group of myocytes subjected to ischemia/reperfusion injury.

3.2 NaHS induced cardioprotection via regulation of intracellular pH

3.2.1 Effect of NaHS on pH_i in the rat ventricular myocytes

As shown in Figure 11A, application of NaHS (an H_2S donor, 100 μM), but not Krebs' solution, induced an obvious decrease in pHi in the isolated ventricular myocytes. The effect of H_2S occurred rapidly after NaHS administration, suggesting that H_2S readily diffused through the cell membrane and influenced pHi. The decrease in pHi by NaHS mimicked the effect of cariporide (7 μM), a selective NHE-1 inhibitor.

The concentration-dependent response is shown in Figure 11B. NaHS at a concentration range from 10 to 1000 μM (yielding approximately 3.3–330 μM H_2S) produced concentration-dependent decreases in pHi in the rat cardiac myocytes. The strongest effect was obtained with NaHS at 100 μM. For this reason, 100 μM NaHS was applied in the subsequent experiments.

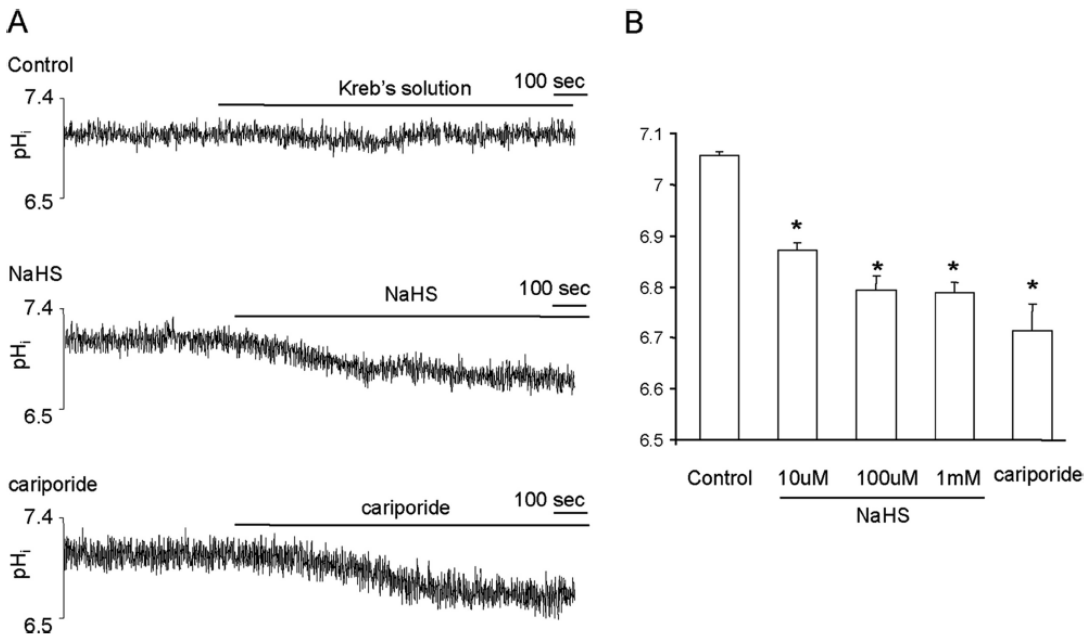

Figure 11 NaHS induces intracellular acidosis in the single cardiac myocyte.

pH_i was measured in cardiac myocytes loaded with BCECF/AM by a dual-wavelength excitation spectrofluorometer. A, representative tracings showing the effect of vehicle (Krebs), NaHS (100 μM), and cariporide (7 μM) on intracellular pH_i. B, pooled data show the effect of NaHS (10, 100, and 1000 μM) and cariporide on pH_i. Results are presented as mean±S.E.M. (n=9–36). *, p<0.001 compared with pH_i in the vehicle-treated myocytes.

3.2.2 Effect of NaHS on NHE-1 activity in rat ventricular myocytes

We had reported previously that U50,488H stimulates NHE-1 activity in cardiac myocytes (Bian, Wang et al. 1998). As shown in Figure 12A, U50,488H at 30 μM significantly increased the pHi in the isolated ventricular myocytes. Both cariporide (7 μM) and NaHS (100 μM) abolished this effect, suggesting that, like cariporide, H_2S may also inhibit NHE-1 activity.

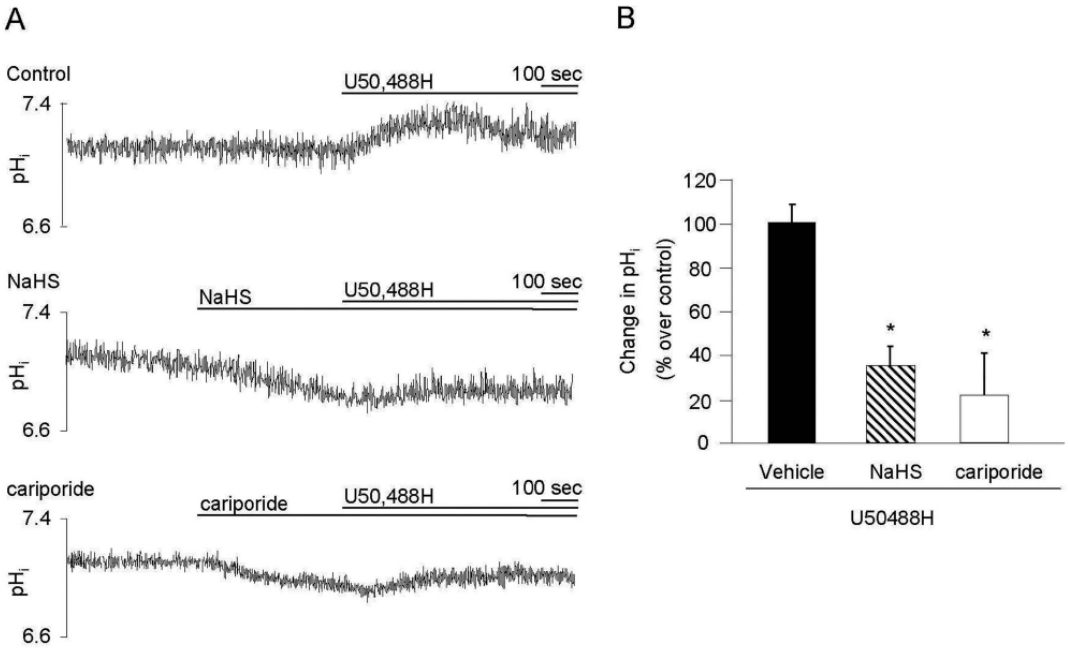

Figure 12 Both NaHS and cariporide abolish the pH regulatory effect of U50,488H.

A, representative tracings demonstrating the effect of NaHS (100 μM) and cariporide (7 μM) on U50,488H-induced intracellular alkalinization. NaHS and cariporide were added 6 min before exposure to U50,488H (30 μM) for another 10 min. B, group data are shown as mean±S.E.M. (n=21–29). *, p<0.001 versus the value of pH_i change in the vehicle group with U50,488H treatment.

To verify the involvement of NHE-1 in the action of NaHS, we determined the activity of NHE-1 in cardiac myocytes by measuring the recovery rate of cells from intracellular acidification (Frohlich and Wallert 1995). Figure 13A shows the representative tracings of pH_i in individual ventricular myocytes subjected to intracellular alkalinization. Transient application of NH_4Cl loads the cell with alkali, thus inducing a significant elevation in pH_i. Subsequent washout with NH_4Cl imposes a strong acid load by trapping protons in the myocytes and thus induces intracellular acidification. As shown in Figure 13B&C, both NaHS and cariporide significantly attenuated the recovery slope of pH_i during washout, and NaHS decreased the NHE-1 activity.

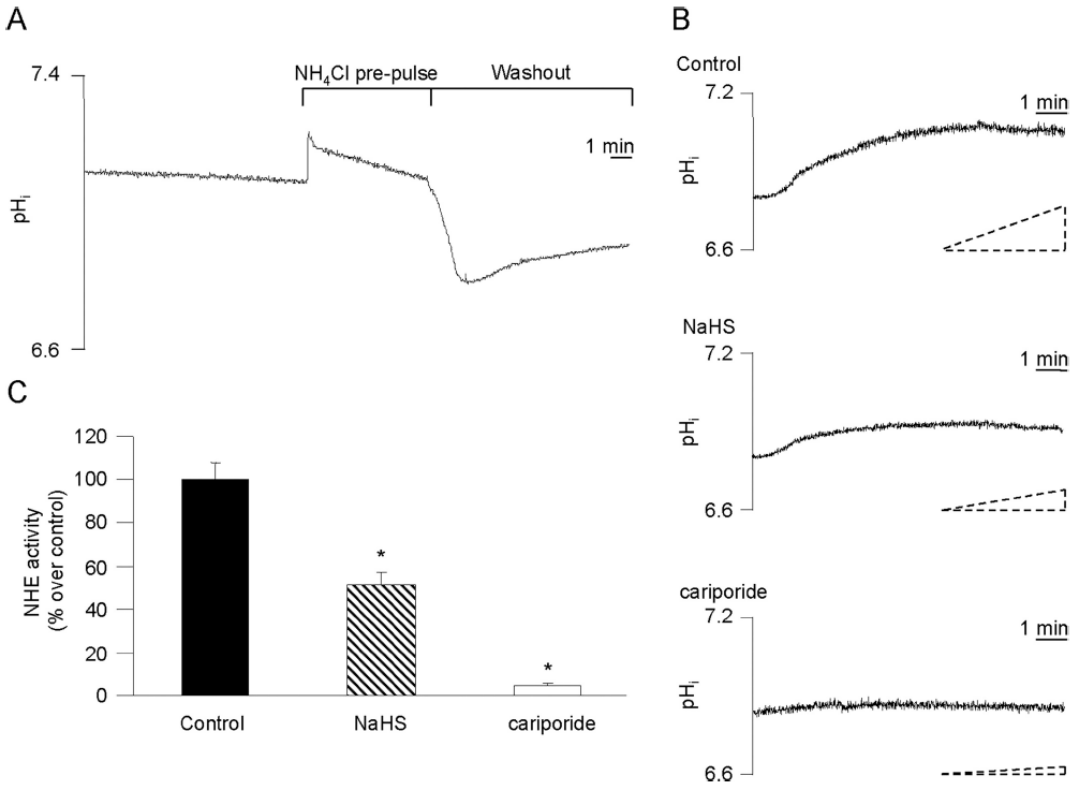

Figure 13 Effect of NaHS on NHE-1 activity in the cardiac myocytes.

A, representative tracing showing the intracellular pH changes with a typical NH_4Cl prepulse method. Myocytes were subjected to intracellular acidosis by transient (6 min) exposure to 20 mM NH_4Cl and subsequent washout and perfused with HEPES-buffered solution for 8 min. The slope of pH_i recovery determines the sarcolemmal NHE-1 activity. B, representative tracings for pH_i recovery from intracellular acidification caused by NH_4Cl washout with and without NaHS or cariporide treatment. The triangles represent the fitted slopes of pH_i recovery, indicative of NHE-1 activity. C, mean value showing that NaHS and cariporide significantly inhibited NHE-1 activity. Mean±S.E.M. (n=22–30). *, p<0.001 versus control.

3.2.3 Effect of NaHS on CBE activity in the isolated ventricular myocytes

Given that CBE is the main acid loader in cardiac myocytes, we also determined its activity by perfusing NH_4Cl in the presence and absence of NaHS. NH_4Cl perfusion is a widely adopted method to measure the capacity of CBE to maintain pH_i (Xu and Spitzer 1994). NH_4Cl loads the cells with alkali, resulting in a significant elevation in pH_i. As an acid loader, CBE may consequently activate and extrude HCO_3^- in exchange for Cl^- which eventually restores the pH_i to normal. Therefore, the slope of the pH_i recovery phase provides a measure of CBE activity. As shown in Figure 14, H_2S inhibits the activity of CBE. Since inhibition of CBE alone induces intracellular alkalization, instead of acidification, these data indicate that the intracellular acidification induced by H_2S was mediated by NHE-1 instead of CBE.

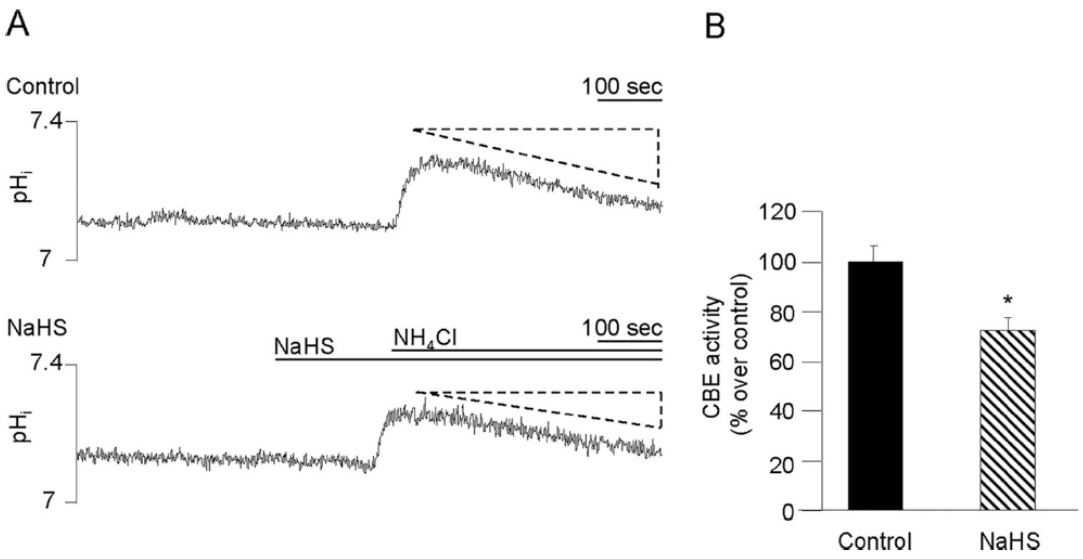

Figure 14 Effect of NaHS on CBE activity in cardiac myocytes.

A, representative tracings for pH_i recovery from intracellular alkalinization caused by the rapid addition of NH_4Cl into bath solution with and without NaHS treatment. The triangles represent the fitted slopes of pH_i recovery, indicative of CBE activity. B, mean value showing that NaHS significantly inhibited CBE activity. Mean±S.E.M. (n=22–24). *, p<0.001 versus the value in the control group.

3.3 The effect of NaHS on NHE-1 activity is mediated by PI3K/Akt and protein kinase G (PKG) pathways

Since activation of PI3K/Akt (Snabaitis, Cuello et al. 2008) or PKG (Perez, Piaggio et al. 2007) inhibits NHE-1 activity, we examined whether the effect of H_2S involves these pathways. As shown in Figure 15A, blockade of PI3K with LY294002 (1 μM) or PKG with KT5823 (0.5 μM), neither of which had a significant effect on NHE-1 activity, produced marked attenuation of the inhibitory effect of NaHS on NHE-1 activity. These data suggest that the activation of PI3K/Akt and PKG may somehow be involved in the inhibitory effect of NaHS on NHE-1 activity.

We then investigated the effect of NaHS on PKG by measuring its activity with a semi-quantitative immunoassay kit. As shown in Figure 15B, NaHS significantly increased the PKG activity in the myocytes subjected to ischemia. Pretreatment with Akt VIII, the Akt inhibitor, but not KR-32568 (KR, 1 μM), an NHE-1 inhibitor, abolished this effect. This implies that activation of Akt by H_2S is upstream to that of PKG.

Moreover, pretreatment with KT5823 for 10 min failed to attenuate NaHS-stimulated Akt phosphorylation (Figure 15C). The similar effect was also observed under ischemic conditions. Ischemia for 30 min significantly down-regulated Akt phosphorylation, which was reversed by NaHS (100 μM) pretreatment for 10 min. Blockade of PKG with KT5823 also failed to attenuate the effect of H_2S on Akt phosphorylation during ischemia (Figure 15D). Taken together, our data clearly suggest that the activation of PKG is secondary to the NaHS-induced Akt activation but

upstream to NHE-1 inhibition.

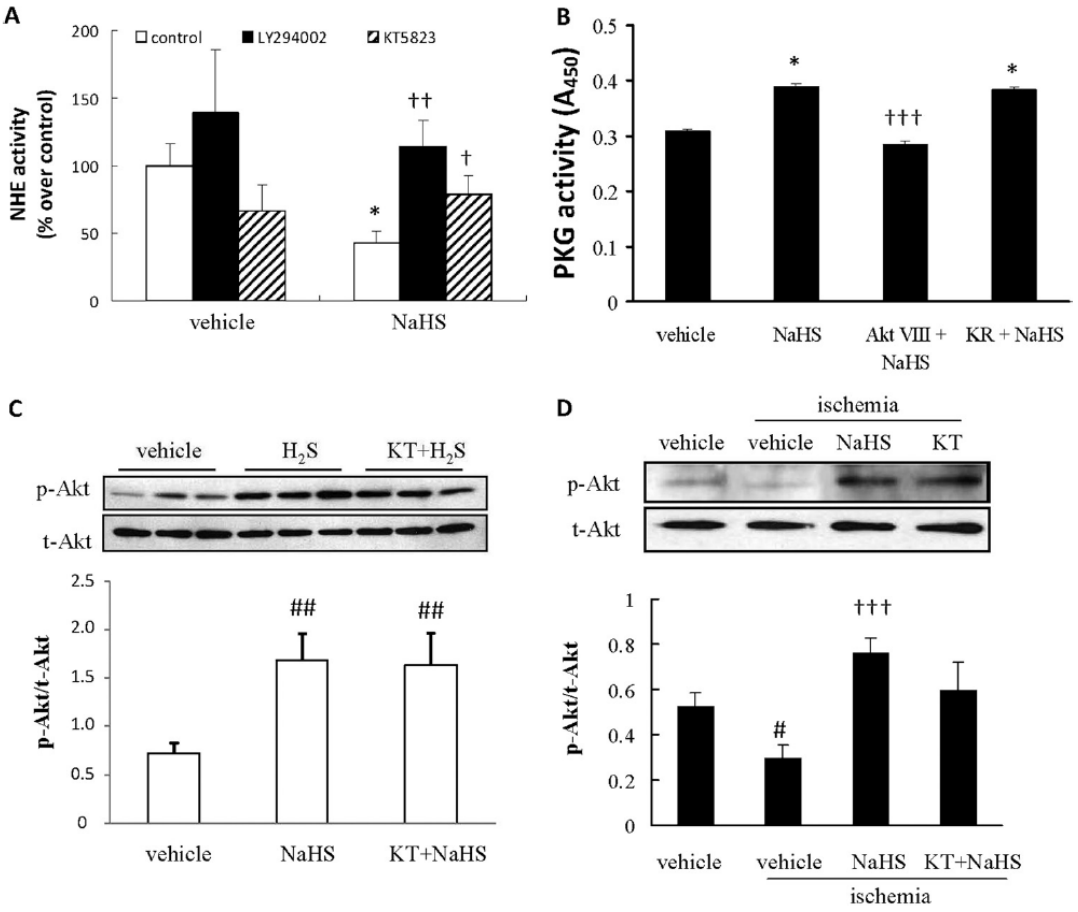

Figure 15 Role of PI3K/Akt and PKG in NaHS-suppressed NHE-1 activity.

A, blockade of PI3K/Akt with LY294002 (1 μM) or PKG with KT5823 (0.5 μM) significantly attenuated the effects of H_2S on NHE-1 activity. Mean±S.E.M. (n=5–9). *, p<0.01 versus control. †, P<0.05; ††, p<0.01 versus the NaHS treated group. B, PKG activity in the isolated cardiac myocytes was determined with a semiquantitative immunoassay kit. Group data were obtained from five independent experiments. *, P<0.01 versus ischemia group. †††, P<0.001 versus NaHS plus ischemia group. C and D, effect of NaHS (100 μM) on Akt phosphorylation in cardiac myocytes in the presence or absence of KT5823 (0.5 μM; KT) in normal (C) and ischemic (D) cardiac myocytes. Pretreatment with KT5823 for 10 min failed to affect the stimulatory effect of NaHS on Akt phosphorylation in cardiac myocytes. The cardiac myocytes were pretreated with NaHS for 10 min and remained in contact with NaHS during ischemia

for 30 min. At the end of ischemia, the myocytes were harvested and subjected to Western blot analysis. The representative gels show the levels of phosphorylated Akt (p-Akt) and total Akt (t-Akt) in the myocytes subjected to various treatments. Mean<S.E.M. (n=8). #, p<0.05; ##, p<0.01 versus vehicle-treated myocytes without ischemia. †††, P<0.001 versus vehicletreated myocytes subjected to ischemia.

We further confirmed that the cardioprotection offered by NaHS is mediated by PI3K/Akt/PKG pathway. As shown in Figure 16, the heart contractile function presented by LVDP and ±dP/dt of the LY+NaHS group could not be restore as that of NaHS group did, indicating that the effect of NaHS on isolated hearts was also alleviated by LY294002 (1 μM, a PI3K inhibitor). However, LY294002 failed to completely block the action of NaHS, as the effect of NaHS on LVeDP was not alleviated at all. As shown in Figure 17&18, similar conclusion can be drawn when Akt VIII (1μM, a specific Akt inhibitor) and KT5823 (0.5 μM, a PKG inhibitor) were administrated to block Akt and PKG respectively, that Akt VIII and KT5823 could only obstruct the action of NaHS on LVDP and ±dP/dt but not on LVeDP. These data suggest that NaHS-induced cardioprotection is, at least partly, mediated by PI3K/Akt and PKG pathways. It is interesting to note that blockade of PI3K/Akt or PKG had no significant effect on NaHS-induced protection on LVeDP. These data imply that the protective effects of NaHS may not be solely mediated by these signaling pathways.

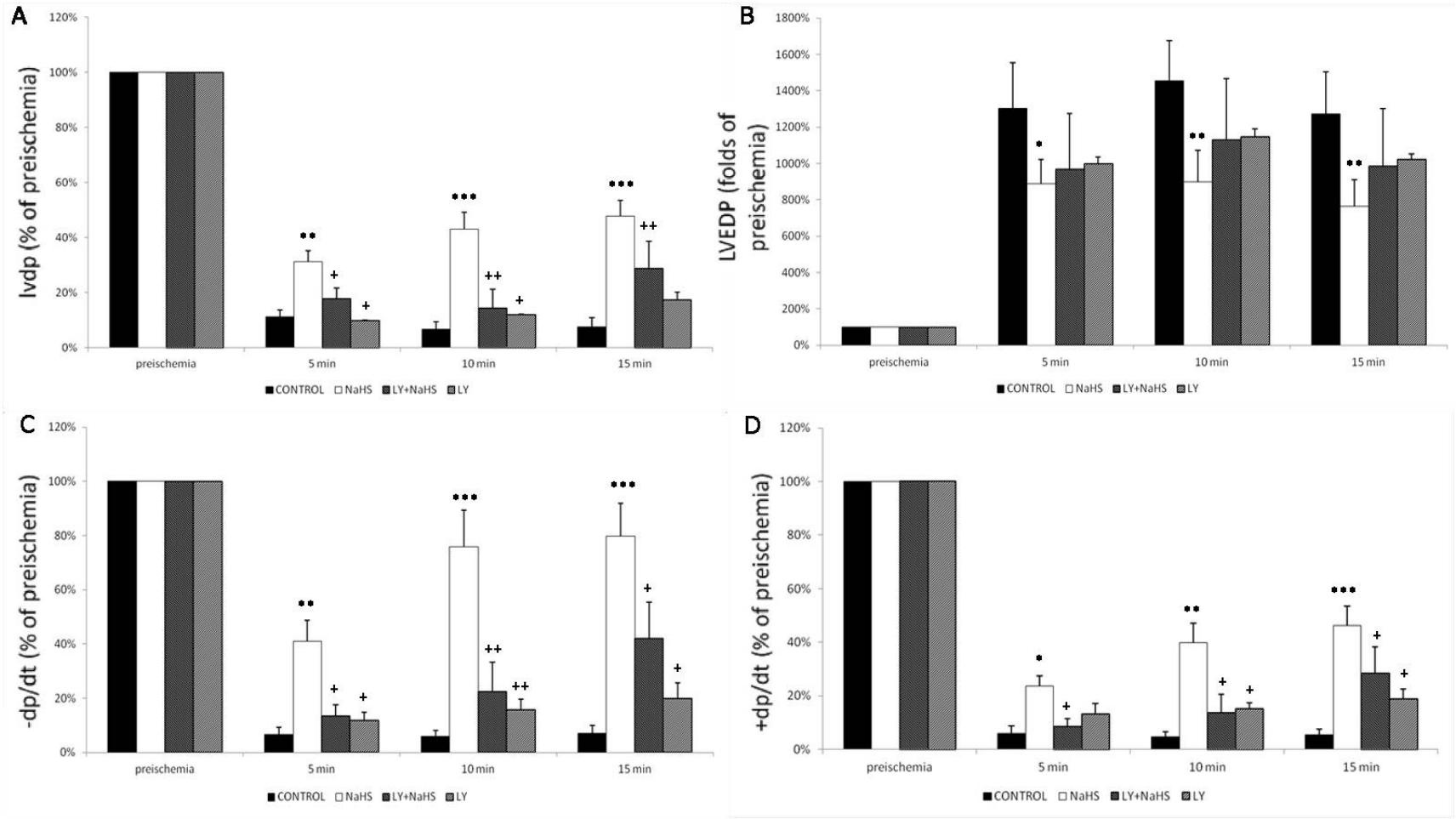
A
lvdp (% of preischemia)
120%
100%
80%
60%
40%
20%
0%
preischemia
5 min
10 min
15 min
CONTROL
NaHS
LY+NaHS
LY
B
LVEDP (folds of preischemia)
1800%
1600%
1400%
1200%
1000%
800%
600%
400%
200%
0%
C
-dp/dt (% of preischemia)
D
+dp/dt (% of preischemia)

Figure 16 LY294002 blocks the cardioprotective effect of H_2S on heart contractile function by inhibiting PI3K activity.

This figure shows the role of PI3K in the cardioprotection of H_2S on heart contractile function in control, NaHS, LY+NaHS, and LY groups. Blockade of PI3K with LY294002 (1 μM), which alone had no effect, significantly attenuated the effects of H_2S on heart contractile function. Inhibitors were given 10 min before the addition of NaHS for 10 min followed by no flow ischemia. Mean<S.E.M. (n = 5–8). *, p<0.05; **, p<0.01; ***, p<0.001 versus the corresponding values in the control group. +, p<0.05; ++, p<0.01 versus the corresponding values in the NaHS-treated group.

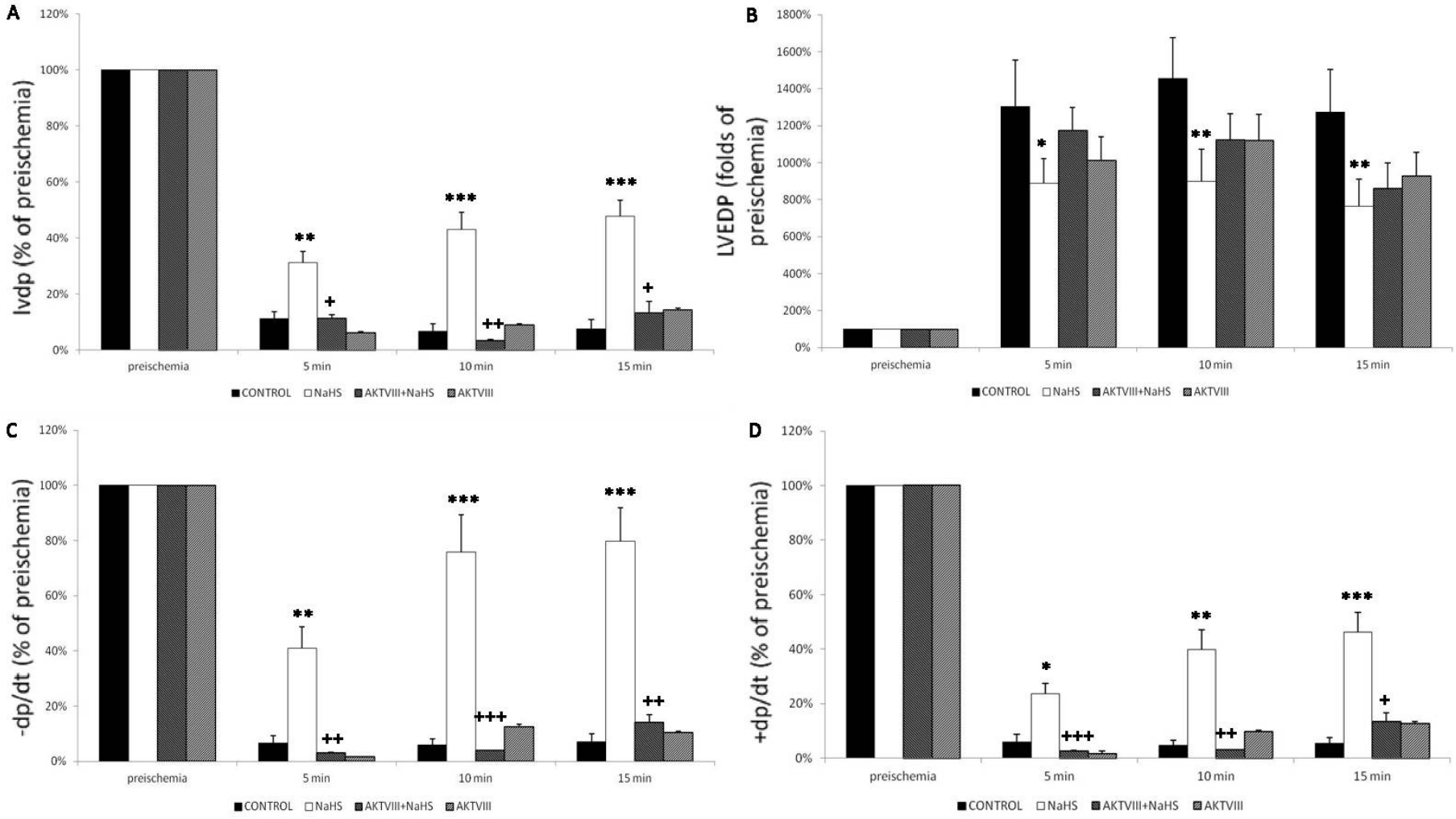
A
lvdp (% of preischemia)
120%
100%
80%
60%
40%
20%
0%
preischemia
5 min
10 min
15 min
CONTROL
NaHS
AKTVIII+NaHS
AKTVIII
B
LVEDP (folds of preischemia)
1800%
1600%
1400%
1200%
1000%
800%
600%
400%
200%
0%
C
-dp/dt (% of preischemia)
D
+dp/dt (% of preischemia)

Figure 17 Akt VIII blocks the cardioprotective effect of H_2S on heart contractile function by inhibiting Akt activity.

This figure shows the role of Akt in the cardioprotection of H_2S on heart contractile function in control, NaHS, AKTVIII+NaHS, and AKTVIII groups. Blockade of Akt with Akt VIII (1 μM), which alone had no effect, significantly attenuated the effects of H_2S on heart contractile function. Inhibitors were given 10 min before the addition of NaHS for 10 min followed by no flow ischemia. Mean<S.E.M. (n = 5–8). *, p<0.05; **, p<0.01; ***, p<0.001 versus the corresponding values in the control group. +, p<0.05; ++, p<0.01 versus the corresponding values in the NaHS-treated group.

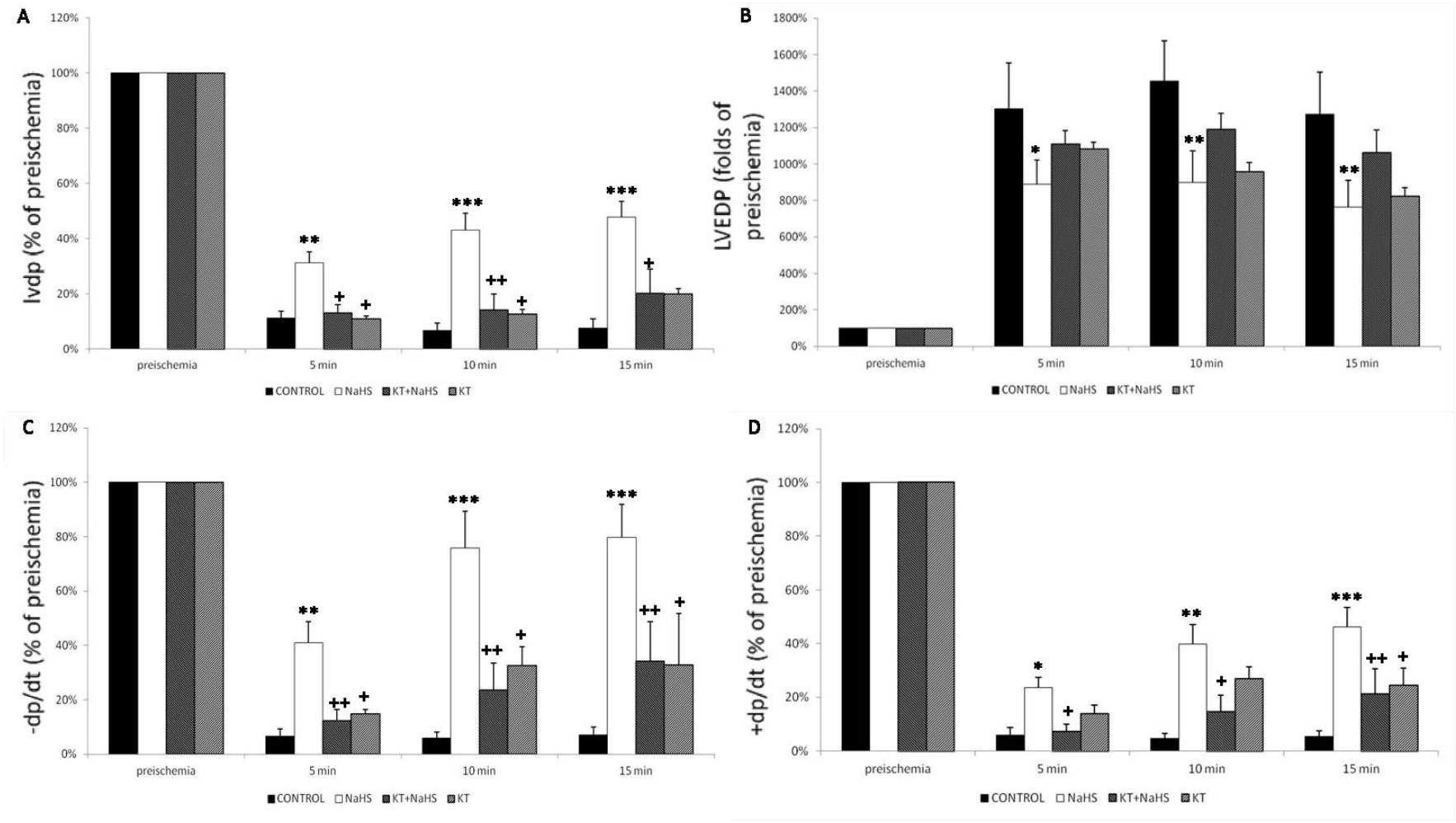
A
lvdp (% of preischemia)
120%
100%
80%
60%
40%
20%
0%
preischemia
5 min
10 min
15 min
CONTROL
NaHS
KT+NaHS
KT
B
LVEDP (folds of preischemia)
1800%
1600%
1400%
1200%
1000%
800%
600%
400%
200%
0%
C
-dp/dt (% of preischemia)
D
+dp/dt (% of preischemia)

Figure 18 KT5823 blocks the cardioprotective effect of H_2S on heart contractile function by inhibiting PKG activity.

This figure shows the role of PI3K in the cardioprotection of H_2S on heart contractile function in control, NaHS, KT+NaHS, and KT groups. Blockade of PKG with KT5823 (0.5 μM), which alone had no effect, significantly attenuated the effects of H_2S on heart contractile function. Inhibitors were given 10 min before the addition of NaHS for 10 min followed by no flow ischemia. Mean<S.E.M. (n = 5–8). *, p<0.05; **, p<0.01; ***, p<0.001 versus the corresponding values in the control group. +, p<0.05; ++, p<0.01 versus the corresponding values in the NaHS-treated group.

CHAPTER 4 DISCUSSION

This study is aimed to investigate the possible mechanism and signaling pathways involved in the cardioprotection, induced by H_2S, the third member of gasotransmitter family, against ischemia/reperfusion.

Previous studies from our and other groups have found that H_2S plays a critical role in protecting the heart against ischemic injury (Geng, Chang et al. 2004; Geng, Yang et al. 2004). In the current study, our data showed that H_2S elicited cardioprotection on hemodynamic function and cell viability during 30min no-flow ischemia and reperfusion. Mechanisms of these results were explored.

In the heart, intercellular pH influences contractility (Vaughan-Jones, Eisner et al. 1987; Kohmoto, Spitzer et al. 1990) and affects the generation of arrhythmias (Orchard and Cingolani 1994; Ch'en, Vaughan-Jones et al. 1998). In the present study, we found that NaHS over a concentration range of 10-1000 μM decreased pH_i in a concentration-dependent manner.

Furthermore, NaHS abolished the U50,488H-stimulated NHE activity. The assay of NHE activity further confirms that H_2S-induced intracellular acidosis is mediated by inhibition of NHE-1 activity. NHE-1 is an important acid extruder in cells. It is well recognized that NHE-1 inhibition may produce cardioprotection. We also found in the present study that both NaHS and NHE-1 inhibitor protected hearts against ischemic injury. However, the combination of two drugs did not produce any additive or synergistic cardioprotective effect. Our study therefore suggests that NaHS produced

cardioprotection via the inhibition of NHE-1 activity.

The signaling mechanism for the action of H_2S on NHE-1 was also investigated. It has been previously reported that activation of Akt phosphorylates NHE-1 and thus inhibits its activity (Snabaitis, Cuello et al. 2008). We therefore tested whether the inhibition of NHE-1 activity caused by H_2S is mediated by PI3K/Akt pathway. We found that NaHS treatment stimulated Akt phosphorylation in both normal and ischemic cardiac myocytes. Our finding is in agreement with a recent report that H_2S protects cardiac myocytes from hypoxia/reoxygenation-induced apoptosis via stimulation of Akt phosphorylation (Yao, Huang et al. 2010). Moreover, blockade of PI3K with LY294002 or Akt with Akt VIII significantly attenuated the inhibitory effect by NaHS on NHE-1 activity and its subsequent cardioprotection. To confirm the contribution of the PI3K/Akt pathway, we also observed the protective action of NaHS on heart contractile function in the presence or absence of Akt VIII, a specific inhibitor of Akt. Like LY294002, Akt VIII also attenuated the effect of H_2S on cardiac contractile function. These data suggest that the cardioprotection of H_2S involves the PI3K/Akt/NHE-1 pathway. Interestingly, recent studies showed that inhibition of NHE-1 may also stimulate Akt (Jung, Lee et al. 2010). These data suggest that activation of Akt and inhibition of NHE-1 may be co-dependent. In addition to Akt activation, Yao *et al* found H_2S also inhibited GSK3β, and subsequently inhibited the opening of mitochondrial permeability transition pore (mPTP) (Yao, Huang et al. 2010). It is also demonstrated that NHE-1 inhibition may produce cardioprotection via phosphorylation and inhibition of GSK-3β (Jung, Lee et al. 2010). These findings

suggest that H_2S may inhibit GSK-3β through inhibition of NHE-1 activity.

NHE-1 activity can also be regulated by PKG. A previous study showed that blockade of PKG restored the suppressed NHE-1 activity to normal (Perez, Piaggio et al. 2007). We therefore examined the involvement of PKG in the actions of NaHS. We found that blockade of PKG with KT5823 attenuated the effect of NaHS on NHE-1 activity and its protective effects on post-ischemia-induced contractile function injury. This implies that PKG may also be involved in the regulation of NHE-1 activity by NaHS. This is verified by the PKG activity assay. In fact, the NaHS treatment enhanced PKG activity in ischemic myocytes. Moreover, the increase of PKG activity could be blocked by the Akt inhibitor, indicating that Akt is upstream of PKG activation. The failure of the PKG inhibitor KT5823 to attenuate the NaHS-stimulated Akt activation again supports the evidence that activation of PKG is secondary to PI3K/Akt activation.

CBE is the major acid loader in the cardiovascular myocyte. Stimulation of this transporter would be expected to "dampen" the ability of the cell to maintain a normal pH_i range and therefore decreases pH_i. For this reason, we also observed the effect of H_2S on CBE activity. We found that H_2S inhibits, but not stimulates CBE. These data exclude the possibility that CBE contributes to the action of H_2S in regulating pH_i in the cardiac myocytes.

The mechanisms for NHE-1 inhibition-triggered cardioprotection have been well studied previously. Ischemia-induced protons leaving the myocytes via NHE-1 during reperfusion may lead to Na^+ loading, which may subsequently induce Ca^{2+} over loading

as Na^+ leaves the cell via the Na^+/Ca^{2+} exchanger. The resultant rise in $[Ca^{2+}]_i$ is believed to trigger Ca^{2+}-activated proteases and phospholipases that cause the cellular damage (Tani 1990; Pierce and Czubryt 1995). In addition, ischemia/reperfusion may also open mPTP. When the mPTP opens, the permeability barrier of the inner membrane becomes disrupted. This will result in mitochondrial swelling and break the outer membrane of mitochondria. The rupture of the outer membrane will lead to the release of proteins in the intermembrane space such as cytochrome c, AIF and other factors that play a critical role in apoptotic cell death. Once released, these factors activate caspase-9, which in turn activates caspase-3. This protease mediates the proteolytic cleavage of a range of proteins responsible for the rearrangement of the cytoskeleton, plasma membrane, and nucleus that are characteristic of apoptosis. A potent inhibitor of mPTP opening is low pH (Halestrap, Clarke et al. 2004). Therefore, in this way, NHE-1inhibition produces cardioprotection.

However, despite their great promise of cardioprotection in extensive preclinical work (Avkiran, Cook et al. 2008), clinical studies with NHE inhibitors were less convincing. It is shown that direct and global NHE inhibition may trigger non-cardiac adverse effects. Compared with NHE inhibitors, H_2S may have better clinical application potential. This is because, apart from inhibition of NHE-1, H_2S also protects the heart via suppression of β-adrenoceptor function, stimulation of various protein kinases and cardioprotective mediators such as PGE2 and nitric oxide (Bian, Yong et al. 2006; Hu, Pan et al. 2008; Pan, Neo et al. 2008; Yong, Pan et al. 2008; Pan, Chen et al. 2009). In addition, H_2S has been found to be useful in treating heart failure

(Calvert, Elston et al. 2010), hypertrophy (Shi, Chen et al. 2007), and renin-dependent hypertension (Lu, Liu et al. 2010). These data suggest that H_2S treatment may provide multiple cardioprotective effects. However, bearing in mind that the NHE inhibitors were not very successful in clinical development for cardioprotection despite the great promise shown in preclinical studies, the present preclinical study does not rule out the possibility of failed clinical application of H_2S in ischemic heart disease. Clinical investigations are thus warranted to test the potential application of H_2S.

In summary, my study shows that H_2S decreases pH_i in cardiac myocytes by inhibiting NHE-1 activity via a PI3K/Akt/PKG-dependent mechanism. This further offers cardioprotective effects against ischemia/reperfusion-induced injury. The unique action of H_2S suggests a potential therapeutic application for H_2S in ischemic condition of cardiovascular system.

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