HYDROGEN SULFIDE,
A CARDIOPROTECTIVE AGENT IN ISCHEMIC HEART

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ABBREVIATIONS

AAR	area at risk
BCA	β-cyano-L-alanine
CABG	coronary artery bypass grafting
CBS	cystathionine β-synthase
$C[Ca^{2+}]_i$	caffeine-induced intracellular Ca^{2+} transient
CNS	central nervous system
CO	carbon monoxide
COX-2	cyclooxygenase-2
CSE	cystathionine γ-lyase
$E[Ca^{2+}]_i$	electrically-induced intracellular Ca^{2+} transient
H_2S	hydrogen sulfide
HSP	heat shock protein
IP	ischemic preconditioning
I/R	ischemia and reperfusion injury
K_{ATP}	ATP-sensitive-potassium channel
LAD	left anterior descending coronary artery
LDH	lactate dehydrogenase
LV	left ventricle
MAPK	mitogen-activated protein kinase
MI	myocardial infarction
mitoK_{ATP}	mitochondrial K_{ATP} channel
mPTP	mitochondrial permeability transition pore
NaHS	sodium hydrosulfide

NCX	Na^+/Ca^{2+} exchanger
NF-κB	nuclear factor-κB
NO	nitric oxide
NOS	nitric oxide synthase
PAG	DL-propargylglycine
PCI	percutaneous coronary intervention
PI-3	phosphoinositide 3 kinase
PKC	protein kinase C
RACK	receptors for activated C kinase
ROS	reactive oxygen species
RyR	ryanodine receptor
sarcoK_{ATP}	sarcolemmal K_{ATP} channel
SERCA	sarco(endo)plasmic reticulum Ca^{2+}-ATPase
SMCs	vascular smooth muscle cells
SR	sarcoplasmic reticulum
SP	H_2S preconditioning
t_{50}	half-decay time
t_{90}	90%-decay time

SUMMARY

Ischemic heart disease is the leading cause of death in the western society and is becoming increasingly a major health problem in developing countries. In the current study, the role of hydrogen sulfide (H_2S) in the cardioprotection against ischemic injury was investigated in both *in vitro* and *in vivo* models.

The effects of endogenous and exogenous H_2S were first examined in isolated adult rat cardiomyocytes. Endogenous H_2S production was found to be suppressed in cardiomyocytes subjected to lethal ischemia. Preconditioning the cells with brief ischemia partly restored endogenous H_2S level. Inhibition of H_2S biosynthesis blocked the early and late cardioprotection induced by ischemic preconditioning, indicating that endogenous H_2S was necessary for the development of both early and late cardioprotection of ischemic preconditioning. To examine the effect of exogenous H_2S, cardiomyocytes were preconditioned with a H_2S donor, NaHS, at concentrations of 10^{-6} to 10^{-3} mol/L. H_2S preconditioning produced a concentration-dependent protection against ischemia-caused cell death, morphology change and impairment on cell function. A time course study showed that H_2S-induced cardioprotection occurred in 2 time windows (early phase, ~1 h, and late phase, 16-28 h) and was effective to counteract different periods of ischemia and reperfusion. The late cardioprotection was blocked in the presence of a sarcolemmal K_{ATP} channel blocker, a nitric oxide synthase inhibitor or a PKC inhibitor, suggesting their involvement in the signaling pathway of H_2S preconditioning. Western blotting analysis confirmed that H_2S preconditioning activated three isoforms of PKC, α, ε and δ. The activated PKC mediated the acceleration of cytosolic Ca^{2+} clearing which in turn prevented cytosolic Ca^{2+} accumulation and myocytes hypercontracture during ischemia and reperfusion.

In a rat model of myocardial infarction, the effect of H_2S was examined *in vivo* with intraperitoneal injection of NaHS. Assessment of infarct size revealed that a single bolus of NaHS administered one day before MI reduced infarct size by 78% at the optimal dose of 1μmol/kg, whereas rats receiving three boluses of NaHS once per day after MI only displayed a maximum reduction of 38% in infarct size. A combined treatment of both preconditioning and post-treatment did not produce stronger protection than that produced by H_2S preconditioning alone. Furthermore, H_2S preconditioning also remarkably reduced LV dilatation and wall thinning, as manifested by LV internal diameter and anterior wall thickness.

In conclusion, the current study has demonstrated that H_2S is a potent cardioprotective agent against ischemic injury. H_2S preconditioning may represent an effective and promising intervention for ischemic heart disease.

Chapter 1 Introduction

1.1 General Overview

Ischemic heart disease, mainly manifested by myocardial infarction, is a syndrome characterized by high mortality, frequent hospitalization and reduced life quality. As a global health problem, ischemic heart disease is cited as the leading cause of death in 13 countries, primarily in US and most European countries (American Heart Association, 2008). In Singapore, ischemic heart disease accounts for 18.5% of total death, ranked as the second most common cause of mortality in 2006 (Ministry of Health, 2006).

Despite remarkable advances in basic science and clinical studies, preventing and reversing ischemic heart disease remains a great challenge to scientists and clinicians working for the health of millions of patients worldwide. In the 20th century, classical physiology studies provided valuable insight into the pathophysiology of myocardial ischemia and reperfusion. Next to reperfusion therapy, preconditioning of the heart with nonlethal ischemic episodes emerged in the late 1980s as a promising means to limit damage by myocardial infarction. It then became clear that the cardioprotective effect of preconditioning is mediated by certain signal transduction molecules. Elucidating the intracellular signal transduction mediated by these molecules allowed the identification of pharmacological agents that can mimic the cardioprotection without ischemia. Studies driven by this particular interest have derived a special field in the cardioprotection called pharmacological preconditioning and translation of the basic science findings from this field into clinical use is yielding encouraging results.

Over the past decades, the focus of research in cardioprotection against myocardial ischemia has shifted from physiology and biochemical studies at whole organ level to molecular studies at organelle and intracellular level. The use of biochemistry, cellular and molecular biology, genomics, and proteomics has become more important to identify the signaling complexes mediating cardioprotection against I/R injury. It is expected that utilization of these technologies will bring a better understanding of the progression of the disease and enable the researchers to develop more effective interventions for ischemic heart disease.

1.2 Ischemic heart disease

1.2.1 Epidemiology

Ischemic heart disease, also called coronary heart disease, is the leading cause of death in western society, claiming hundreds of thousands of lives each year. In the United States, for instance, some 8,700,000 men and 7,300,000 women are living with ischemic heart disease (American Heart Association, 2008). Every year, an estimated 920,000 people suffer a new or recurrent coronary attack, and about 38% of them die as a result of the attack (American Heart Association, 2008). In the developing countries, the death rate from ischemic heart disease is third to AIDS and lower respiratory infections (CGIRS, 2006). The World health organization (WHO) predicts that ischemic heart disease will cause 11.1 million deaths globally in 2020, becoming the top killer of humans in the whole world (World Health Organization web site, *www.who.int/ncd/cvd*).

1.2.2 Risk factors

Atherosclerosis is a major cause of ischemic heart disease. The risk factors for atherosclerosis are generally those for ischemic heart disease. Extensive clinical and

statistical studies have identified several factors that significantly increase the risk of coronary heart disease (American Heart Association web site, *http://www.americanheart.org/presenter.jhtml?identifier=4726*). Some of the factors cannot be changed, including older age, male gender, race and heredity, while many others are modifiable, like tobacco smoke, high blood cholesterol, high blood pressure, physical inactivity, obesity and diabetes mellitus, all of which can be treated or controlled either by changing lifestyle or taking medicine.

1.2.3 Pathology

Myocardial infarction (MI) is a common presentation of ischemic heart disease. A myocardial infarction occurs when an atherosclerotic plaque that slowly builds up in the lumen of a coronary artery suddenly ruptures and blocks the blood flow downstream. The formation of atherosclerotic plaque is a chronic inflammatory response in the walls of arteries, in large part due to the accumulation of white blood cells and low density lipoproteins (LDL, the proteins carrying cholesterol and triglycerides). When LDL gets through an artery, oxygen free radicals react with it and form oxidized-LDL, which then attracts macrophages and T-lymphocytes. After these white blood cells take up large amounts of cholesterol, they are called foam cells. When foam cells die, their contents are released, which attracts more macrophages and creates an extracellular lipid core in the inner surface of atherosclerotic plaque. Conversely, the outer, older portions of the plaque become calcified and stiffer over time. After decades of progression, these plaques may rupture, activate the blood clotting system and lead to the formation of a thrombus, which obstructs blood flow acutely. Upon the obstruction, downstream myocardium is starved of oxygen and nutrients, where myocardial infarction develops. Most individuals with

coronary heart disease show no evidence of narrowed artery for decades until the disease progresses to the advanced state when the first symptom, often a "sudden" heart attack, finally arise (American Heart Association, 2008).

The myocardium can tolerate brief periods (up to 15 minutes) of severe myocardial ischemia without resulting cardiomyocyte death (Buja, 1998). If impaired blood flow lasts more than 20-30 minutes, it will usually initiate irreversible cell injury (infarction). With increasing duration of ischemia, greater cardiomyocyte damage could develop upon a re-established blood flow to the blocked heart area, termed reperfusion injury (Yellon and Baxter, 2000). The intracellular events of ischemia and reperfusion injury will be discussed in detailed in the session 1.2.6 “Myocardial ischemia and reperfusion injury”.

1.2.4 Clinical features and diagnosis

The diagnosis of cardiac ischemia is usually made by integrating the symptoms and the results from physical examinations with biomarker, electrocardiography (ECG) or other imaging techniques (Antman et al., 2004). Chest pain is the most common symptom of acute myocardial infarction and is often described as a sensation of tightness, pressure, or squeezing. Chest pain due to ischemia of the heart muscle is termed angina pectoris.

Cardiac biomarkers are proteins released into the bloodstream from the damaged myocytes, such as myoglobin, cardiac troponin T and I, creatine kinase (CK), lactate dehydrogenase (LDH) and so on. Myocardial necrosis can be recognized when blood levels of specific biomarkers are increased. Disproportional elevation of the MB subtype of the enzyme CK was found very specific for myocardial injury. Elevated troponins in the setting of chest pain may also indicate a high likelihood of a myocardial infarction (Aviles et al., 2002).

Electrocardiography plays an important role in the diagnosis of patients with suspected myocardial infarction (Thygesen et al., 2007). The acute or evolving changes in the ST-T waveforms and the Q-waves allow the clinician to date the event, suggest the infarct-related artery and estimate the amount of myocardium at risk. The earliest manifestations of myocardial ischemia are typical T waves and ST segment changes. As the myocardial infarction evolves, there may be loss of R wave height and development of pathological Q waves.

Non-invasive imaging is also useful in diagnosis and characterization of myocardial infarction with the ability to detect wall abnormities (Thygesen et al., 2007). Commonly used imaging techniques in acute and chronic infarction are echocardiography, radionuclide ventriculography and magnetic resonance imaging (MRI). Echocardiography is an excellent real-time imaging technique with strength in the assessment of wall thickness, thickening and motion at rest. Radionuclide imaging allows viable myocytes to be visualized directly, presenting the only commonly available direct method of assessing viability. Cardiovascular MRI is well-validated standard for the assessment of myocardial function with high spatial resolution and moderate temporal resolution. It is, however, more cumbersome and less used in an acute setting.

1.2.5 Complications

Life-threatening complications may occur immediately following the heart attack. These include pulmonary congestion, ventricular rupture, arrhythmia, pericarditis and cardiogenic shock (Antman et al., 2004). A chronic remodeling progress may also start from the injured site and ultimately leads to heart failure.

1.2.6 Myocardial ischemia and reperfusion (I/R) injury

1.2.6.1 Cellular injury

Intensive investigation over decades has provided a detailed understanding of the complexity of the response of myocardium to an ischemic insult. Myocardial ischemia results in a characteristic pattern of metabolic and ultrastructural changes that lead to irreversible injury. Upon the interruption of oxygen supply, mitochondrial oxidative phosphorylation rapidly stops, resulting loss of the major source of ATP production for energy metabolism. A compensatory increase in anaerobic glycolysis for ATP production leads to the accumulation of hydrogen ions and lactate (Buja, 2005). The resultant intracellular acidosis causes alterations in ion transport in the sarcolemma and organellar membranes (Buja et al., 1988) (Thandroyen et al., 1992). Initially, there is increased K^+ efflux related to an increased osmotic load due to the accumulation of metabolites and inorganic phosphate. With a substantial decline in ATP, the Na^+, K^+-ATPase is inhibited, resulting in a further decline of K^+ and an increase in Na^+. Intracellular acidosis also activates the sarcolemmal Na^+–H^+ antiport (Yellon and Baxter, 2000; Karmazyn, 1999), which facilitates proton extrusion in exchange for Na^+. The accumulated Na^+ in turn activates Na^+–Ca^{2+} exchanger which extrudes Na^+ and brings in Ca^{2+}. The resultant cytosolic loading of Ca^{2+} not only induces sustained impairment on contractile function, but also mediates the damage on cell membrane, which leads to the progression of the injury to an advanced stage (Buja, 1991).

The increase in cytosolic Ca^{2+} activates Ca^{2+}–dependent protease and phospholipase which degrades phospholipid and releases lysophospholipids and free fatty acids. Peroxidative stress continuously mounts with accumulation of toxic oxygen species and

free radicals generated from myocytes, endothelial cells, and activated leukocytes, inducing further damage to the membrane phospholipid. Activated proteases cleave cytoskeletal filaments, which disrupts the cellular scaffolds. These changes collectively lead to a loss of membrane integrity and terminally demolish the cell structure.

1.2.6.2 Necrosis and apoptosis of cardiac cells

Necrosis and apoptosis represents the two fundamental forms of cell death: cell injury with swelling, known as necrosis, and cell injury with shrinkage, known as apoptosis (Majno and Joris, 1995). Necrosis occurs when cells are exposed to extreme stumili like ischemia and ends with total cell lysis. Due to the plasma membrane breakdown, cytoplasmic contents including lysosomal enzymes are released into the extracellular environment. Therefore, necrotic cell death is often associated with massive tissue damage and an intense inflammatory response. Apoptosis, in contrast, can occur under physiological or pathological conditions. Cells undergoing apoptosis feature partition of cytoplasm and nucleus into membrane bound-vesicles (apoptotic bodies) which contain ribosomes, morphologically intact mitochondria and nuclear material. Thus, no inflammatory response is elicited *in vivo* due to efficient removal of apoptotic bodies by macrophages or adjacent epithelial cells.

Extensive investigation has pointed to necrosis as the chief form of cardiomyocyte death during ischemic injury. However, more recently, a role of apoptosis has been identified in many forms of cardiac pathology, including myocardial ischemia (Buja and Entman, 1998). The rate and magnitude of ATP depletion are determinants of whether cell injury progresses by apoptosis or necrosis because apoptosis is an ATP-dependent process (Buja, 2005). Generally, in the central region of the infarct, necrosis

predominates, while in the peripheral areas there is a mix of necrosis and apoptosis. Despite little acute cell death in the remote myocardium, increasing levels of both forms of cell death are noted during the late phases (Mani K, 2008). As the severity of ischemia declines and reperfusion supervenes, the quantum of necrosis decreases, while that of apoptosis increases (Anversa et al., 1998).

1.2.6.3 Reperfusion injury

Reintroduction of coronary flow to the infracted area is necessary to resuscitate the ischemic myocardium and limit the extent of myocardial necrosis. However, the effects of reperfusion are complex and may include some deleterious consequences collectively referred to as reperfusion injury (Yellon and Baxter, 2000). This reperfusion injury is manifested by myocardial stunning, microvascular dysfunction and expedition of cell death in certain critically injured myocytes. The major mediators of reperfusion injury are free oxygen radicals, overloaded calcium, and neutrophils (Carden and Granger, 2000; Granger, 1999; Park and Lucchesi, 1999). The oxygen radicals are generated by injured myocytes and endothelial cells, as well as neutrophils activated on reperfusion. Free radicals exacerbate membrane damage and stimulate vasoconstriction, which, when severe enough, cause a “no-flow” phenomenon. Impaired intracellular calcium homeostasis also plays an important role in the reperfusion injury. The overloaded calcium induces maximum contraction of the myofibrils upon reperfusion, resulting in a disruptive type of necrosis, termed contraction band necrosis (Verma et al., 2002). An increase in mitochondrial [Ca^{2+}] triggers the opening of mitochondrial permeability transition pore (mPTP) and leads to the release of cytochrome C and other proapoptotic factors that initiates the apoptotic cascade (Halestrap et al., 2004). Reperfusion is also a

potent stimulus for neutrophil activation and accumulation, which in turn serve as potent stimuli for reactive oxygen species production. The neutrophils accumulate in the microcirculation, release inflammatory mediators, and contribute to microvascular obstruction and the no-reflow phenomenon.

1.3 Interventions for cardiac ischemia

The search for approaches to protect the heart against ischemia during coronary occlusion has been going on for half a century in both clinical settings and basic research. In the current session, we will describe these approaches from the earliest efforts to limit myocardial infarct size to the cutting edge of stem cell therapy.

1.3.1 Clinical Treatment

1.3.1.1 First line

Myocardial infarction is a medical emergency which demands immediate attention and activation of the emergency medical services. Oxygen, aspirin (antiplatelet drug), glyceryl trinitrate (prodrug of NO) and morphine (analgesia), hence the popular MONA (morphine, oxygen, nitro, aspirin), are the first line drugs recommended to be administered as soon as the symptoms occur (Antman et al., 2004). Once diagnosed as myocardial infarction, the patient is often given other pharmacologic agents, including beta blockers, anticoagulation (typically with heparin), and possibly additional antiplatelet agents such as clopidogrel (Antman et al., 2004). These agents are typically not started until the patient is evaluated by an emergency room physician or under the direction of a cardiologist.

1.3.1.2 Reperfusion therapy

The ultimate goal of the management in the acute phase of myocardial infarction is to salvage as much myocardium as possible and prevent further infarction. Timely reperfusion of coronary flow facilitates cardiomyocyte salvage and decreases cardiac cell death. Modalities for reperfusion include thrombolysis, percutaneous coronary intervention (PCI) and coronary artery bypass grafting (CABG).

Thrombolytic therapy achieves reperfusion by lysing the thrombi in the infarct artery. The effectiveness of thrombolytic therapy is determined by the timing of administration of thrombolytic agents. The best results are always observed when the thrombolytic agent is used within two hours of the onset of symptoms (Boersma et al., 1996). After 12 hours, associated risks like intracranial or systemic bleeding outweigh any benefit (Late, 1993). An ideal thrombolytic drug would lead to rapid reperfusion, have a high sustained patency rate, be specific for recent thrombi, be easily and rapidly administered, and create a low risk for intra-cerebral and systemic bleeding (White and Van de Werf, 1993). Currently available thrombolytic agents are streptokinase, urokinase, and alteplase (recombinant tissue plasminogen activator).

Percutaneous coronary intervention (PCI), commonly known as coronary angioplasty or simply angioplasty, is a surgical procedure to treat the blocked coronary arteries by inflating a balloon within the artery to crush the thrombus. The procedure involves performing a coronary angiogram to determine the location of the blocked vessel, followed by balloon angioplasty to compress the plaque, and implantation of stents to prop the vessel open. The benefit of a prompt expertly-performed PCI over thrombolytic therapy has been well established (Keeley et al., 2003; Grines et al., 1993). However,

logistic and economic obstacles seem to hinder a more widespread application of PCI (Boersma et al., 2006).

Coronary artery bypass graft surgery is another important approach to improve the blood supply to the blocked myocardium. During the surgery, an artery or vein from elsewhere in the patient's body is grafted to the coronary artery to bypass narrowings or occlusions. Several arteries and veins can be used; however the left internal thoracic artery, usually grafted to the left anterior descending coronary artery (LAD), have been demonstrated to last longer than great saphenous vein grafts (Raja et al., 2004). Emergency CABG is less common than PCI for the treatment of an acute myocardial infarction. However, in patients with two or more coronary arteries affected, bypass surgery is superior to PCI in terms of long-term survival rates (Hannan et al., 2005).

Because irreversible injury occurs within 2–4 hours of the infarction, there is a limited time window for reperfusion to produce beneficial results. If attempts to restore the blood flow are initiated after a critical period of only a few hours, the result is reperfusion injury instead of amelioration (Faxon, 2005). Moreover, reperfusion is unable to reverse the developed tissue damage. The lost cardiomyocytes will be replaced by a collagen scar that is not contractible and permanently impairs the pump function. Accordingly, intense interest has been directed to investigate the application of stem cell on the repair of heart damage. The present course of this pioneering work will be briefly reviewed in the following session.

1.3.1.3 Stem cell therapy under investigation

It is traditionally hold that the heart muscle itself has no housekeeping mechanism to repair any minor damage, given that the number of myocytes undergoing proliferation is

too low if myocytes proliferation were to act as an effective repair mechanism. Stem cell, with its ability to self-renew and to form any fully-differentiated cell of the body, provides the possibility of repairing end organ damage, particularly the heart that has undergone myocardial infarction.

Several studies have suggested that bone marrow derived progenitor cells were able to repair the hearts of animals after myocardial injury (Tomita et al., 1999; Toma et al., 2002; Orlic et al., 2001). In one report, bone-marrow-derived cells were injected directly into the heart of the mouse after myocardial infarction was induced (Orlic et al., 2001). Newly formed myocardium composed of proliferating myocytes and vascular structures was found to occupy 68% of the infarcted portion of the ventricle 9 days after transplanting the bone marrow cells. Using immunofluorescence techniques the investigators showed that these primitive bone-marrow-derived cells had undergone a process of differentiation that led them to express various markers specific to cardiomyocytes. This is supported by several other works suggesting that adult stem cells, in particular those derived from bone marrow, were capable of targeting the site of myocardial injury as well as undergoing differentiation into cardiomyocytes (Jackson et al., 2001; Beltrami et al., 2003).

These initial findings in animal models have prompted a series of clinical studies in human beings. Several groups have independently reported improvement in cardiac function in patients treated with stem cells derived from their own bone marrow after a myocardial infarction (Assmus et at., 2002; Strauer et al., 2002; Wollert et al., 2004). The delivery route of progenitor cells included intracoronary, percutaneous intramyocardial and direct intramyocardial at the time of coronary artery bypass graft. Importantly, most

of these studies reported few side-effects. However, a failure to explain the action mechanism underlying the improvement in cardiac function has provoked concerns. Moreover, these trials so far have not been double-blind randomized. Further definitive clinical studies are essentially necessary, especially randomized controlled trials (Mathur and Martin, 2004). The benefit of this novel approach still needs to be confirmed and optimized before it can be applied to treating patients with ischemic heart disease.

1.3.2 Ischemic Preconditioning (IP)

With exception of reperfusion therapy, most early attempts to salvage the myocardium during acute myocardial infarction have failed to directly reduce infarct size (Przyklenk and Kloner, 1998). The first indication that the heart can adapt itself after repeated ischemic stress was demonstrated in porcine myocardium, where lactate release in a subsequent ischemia/reperfusion (I/R) episode was significantly lower compared with lactate release in the first episode of I/R (Verdouw et al., 1979). In 1986 Murry and his colleagues published a landmark article in which they documented that four repetitive 5-min of regional ischemia induced an extremely powerful protection against a subsequent lethal ischemia in anesthetized dogs. Infarct size was limited to 25% of that seen in the control group after 40 min of sustained ischemia (Murry et al., 1986). The investigators named this cardiac warm-up phenomenon as "ischemic preconditioning" (IP). Subsequently, numerous studies using various models (e.g., liver, kidney, brain, and endothelial cells) showed that short period(s) of ischemia or anoxia could allow tissues to survive subsequent ischemia that would have otherwise been lethal (Sanada and kitakaze, 2004). Understanding this natural protection has since become one of the major targets in search for preventions against ischemic damages.

While initial studies demonstrated that ischemic preconditioning could protect the heart against sustained ischemia that occurred soon after preconditioning, Kuzuya et al. (Kuzuya et al., 1993) and Marber et al. (Marber et al., 1993) independently reported in 1993 that the cardioprotective effect of ischemic preconditioning was still detectable 24 hours after preconditioning. Kuzuya's group (Kuzuya et al., 1993) also found that the infarct-limiting effect of preconditioning was lost between 3 and 12 hours after a brief period of ischemia, which suggested that there were two separate periods of cardioprotection afforded by ischemic preconditioning. They named them the ''first window'' and ''second window'' respectively. The first window of the protection, often referred to as classical or early phase, develops as early as few minutes after the preconditioning stimulus and lasts only 1–2 hours (Murry et al., 1986). The second window, also known as the late or delayed phase, develops more slowly, 12–24 hours after the preconditioning stimulus, but lasts for 3–4 days (Kuzuya et al., 1993) (Marber et al., 1993). The mechanisms of these two phases are different. The first phase of protection is initiated by posttranslational modifications of proteins that are already present, whereas the second phase is mediated by synthesis of *de novo* proteins (Bolli, 2000); The early phase depends on reactions that occur very rapidly, such as activation of ion channels or phosphorylation of enzymes, whereas the late phase involves processes that take far longer to occur such as modulation of the genes regulating channel proteins, receptor, enzymes, molecular chaperon proteins, or immune factors (Sanada and kitakaze, 2004). However, these two types of cardioprotection seem to share certain triggers, mediators, and effectors despite differences in the timing of participation in each cascade.

1.3.2.1 Cellular mechanisms of the early phase of IP

1.3.2.1.1 Adenosine, bradykinin and opioids

In 1991, Liu et al. (Liu et al., 1991) first discovered that stimulation of the G_i-coupled adenosine A1 receptor was necessary to trigger IP's protection. They used a rabbit model to show that administration of adenosine receptor antagonist, 8-psulfophenyl theophylline (8-SPT), prior to sustained ischemia was able to abolish the protective effect of IP. Infusion of adenosine or A1-specific agonist reproduced the protection afforded by IP. Liu proposed that endogenous adenosine released during IP results in the preconditioned phenotype. Two other endogenously released trigger substances, bradykinin (Wall et al., 1994) and opioids (Schultz et al., 1995), were subsequently found to be involved in IP and appeared to work in parallel. Inhibition of any one of these three receptors blocked IP's protection from a single preconditioning cycle. However, the protection could again be realized if the number of preconditioning cycles was increased. This led Goto et al. (Goto et al., 1995) to suggest that the three receptors had an additive effect required to reach a hypothetical protective threshold. Increase in brief ischemia/reperfusion cycles released more trigger substances so that two receptors could eventually reach the protective threshold even when the third one was inhibited.

1.3.2.1.2 Protein Kinase C (PKC)

The multiple trigger theory mentioned above requires that all triggers converge on a common target. In 1994, Ytrehus et al. (Ytrehus et al., 1994) found that inhibition of PKC abolishes the protection induced by ischemic preconditioning or by pretreatment with adenosine. It was later discovered that the cardioprotection afforded by two other trigger substances, bradykinin (Goto et al., 1995) and opioids (Miki et al., 1998), could also be

blocked by PKC inhibitors. These results suggest that PKC is a strong candidate for the common target and plays a pivotal role in the IP's cardioprotection.

The PKC family consists of 12 closely related Ser/Tre kinases, classified into three distinctive subfamilies. Classical PKCs (cPKC) include PKC α, β_1, β_2 and γ isoforms, and require both Ca^{2+} and lipids (i.e. phosphatidylserine, PMA and/or diacylglycerol) for their activation. Novel PKC isoforms (nPKC), which include PKC δ, ε, η, θ and μ, are not sensitive to calcium, but still require lipids for their activation. The subfamily of atypical PKC isoforms, PKC ζ and λ, are not activated by Ca^{2+}, diacylglycerol or PMA and their regulation is more complicated. The number and levels of PKC isozyme expression varies in different tissues and species and changes with developmental stage of the animal.

Early studies on the role of PKC in ischemic preconditioning mainly relied on the pharmacological manipulation with PKC inhibitors and stimulators. In an *in vivo* study performed by Speechly-Dick and colleagues (Speechly-Dick et al., 1994), the PKC inhibitor chelerythrine, administered after a preconditioning stimulus, abolished protection conferred by IP, and caused an increase in infarct size. In the same study, PKC stimulator 1,2-dioctanoyl-*sn*-glycerol, administered prior to sustained ischemia, significantly reduced infarct size.

A large body of data supporting the PKC hypothesis has been obtained in the isolated rat heart subjected to sustained global ischemia (Mitchell et al., 1995; Hu and Nattel, 1995). In this model, functional recovery during reperfusion is typically chosen as the index of damage caused by sustained ischemia, and to evaluate the protective effect of IP. For example, Mitchell et al. showed that preconditioning with brief episode of I/R

significantly increased functional recovery at 40 min after relief of a 20 min period of global ischemia (Mitchell et al., 1995). The PKC inhibitors chelerythrine and staurosporine abolished this protective effect, while the diacylglycerol analogue 1-stearoyl-2-arachidonoyl glycerol mimicked the benefits of ischemic preconditioning. Moreover, immunoblotting for PKC isoforms showed that two major isoforms in rat heart, PKCδ and PKCε, both translocated during brief episodes of transient ischemia from cytosol into membrane and nuclear compartments.

The role of PKC in ischemic preconditioning has been evaluated in a diverse array of models, species and protocols. Most of the results were obtained using pharmacological approach, i.e. by administering PKC inhibitors and/or activators. Unfortunately, these inhibitors or activators are isoform-nonspecific, which makes it difficult to single out the isoforms that are responsible for the IP cardioprotection. Immunoblotting represents an alternative to pharmacological and biochemical methods. By assessing subcellular distribution of PKC isoforms, the involvement of a given isoform can be determined. A better solution is to develop isoform-specific inhibitors and activators. In a study conducted by Gray et al., isoform-specific inhibition of PKCε has been successfully employed. In a cell culture model of hypoxic preconditioning, they found that specific inhibitor of PKCε (i.e. ε-VI-2 peptide) abolished the protection induced by hypoxic preconditioning and phorbol ester (Gray et al., 1997). This isoform specific modification allows researchers to identify the particular isoforms that are necessary for the cardioprotection to occur.

1.3.2.1.3 ATP-sensitive-potassium channel (K_{ATP})

Investigation of K_{ATP} channels has a longer history than studies on ischemic preconditioning. In 1983, Noma (Noma, 1983) first reported the existence of these channels in the myocardium. The cardiac K_{ATP} channel is composed of four Kir6.2 subunits (inwardly rectifying potassium channel) and four SUR2A subunits (sulfonylurea receptor) and is modulated by Mg^{2+} and ATP (Snyders, 1999). Opening of the surface or sarcolemmal K_{ATP} channel (sarcoK_{ATP}) was proposed to produce cardioprotection via shortening of phase 3 repolarization of the cardiac action potential and membrane hyperpolarization, both of which would lead to reduced calcium overload during ischemia/reperfusion and a preservation of ATP (Noma, 1983). These phenomena resemble the acute cardiac responses and cardioprotection afforded by ischemic preconditioning. Indeed, a number of early studies have presented strong evidence for a role for sarcoK_{ATP} in mediating IP, with infarct size reduction as the end point to describe the cardioprotective effect. These include those reported by Yao and Gross (Yao and Gross, 1994) and Schulz et al. (Schulz et al., 1994), who showed an association between action potential shortening and IP, and the study of Haruna et al. (Haruna et al., 1998), who showed digoxin, an inhibitor of Na^{+}-K^{+} ATPase, blocked the cardioprotective effect of IP by indirectly desensitizing K_{ATP} channels. Another study using molecular techniques shed some light on the role of K_{ATP} in alleviating calcium overload during ischemia. Jovanovic et al. (Jovanovic et al., 1986) found that in K_{ATP}-deficient COS-7 cells, marked calcium loading occurred when these cells were exposed to 3 minutes of chemically-induced hypoxia. However, when they cotransfected the cells with both subunits of the sarcoK_{ATP} channel, SUR2A and Kir6.2, the addition of the K_{ATP} opener

pinacidil attenuated the calcium loading. Similar results were obtained with pinacidil in cardiac myocytes expressing the native sarcoK_{ATP} channel.

In 1991, Inoue et al. (Inoue e tal., 1991) found that not only the cell membrane but also the inner mitochondrial membrane possessed ATP-sensitive inward rectifier activity, and they suggested the existence of "mitochondrial K_{ATP} channels" (mitoK_{ATP}). It is thought that a beneficial effect may result from K^+ entry through mitoK_{ATP} and intramitochondrial depolarization. This effect would reduce mitochondrial calcium overload and cause moderate matrix swelling, which leads to slowing of ATP synthesis and accelerated mitochondrial respiration (Holmuhamedov et al., 1998). Moreover, the reactive oxygen species (ROS), which are transiently generated by opening of the mitoK_{ATP} channels, is able to activate downstream cascades and confer the preconditioning effect (Pain et al., 2000). The mitoK_{ATP} could thus be involved in acute IP as either a trigger or an end effector, or both.

Thereafter, the contribution of mitochondrial and sarcolemmal K_{ATP} channels to IP-induced cardioprotection has been studied extensively. The effect that had been considered to be related to sarcolemmal K_{ATP} channels, were found to be actually mediated by mitochondrial K_{ATP} channels (Liu et al., 1999). However, some concerns have been raised over those studies on the role of mitoK_{ATP} channels in IP. Firstly, the structure of mitoK_{ATP} is still largely unknown. It is now thought that mitoK_{ATP} might not include the Kir 6.1 or Kir 6.2 subunits, which are common to sarcolemmal and other K_{ATP} channels (Suzuki et al., 2002). Secondly, the only tool drugs available to pharmacologically modulate mitoK_{ATP} channels (diazoxide as an opener and 5-hydroxydecanoate [5-HD] as an inhibitor) also have direct effects on cellular respiratory

metabolism (Dzeja et al., 2003; Hanley et al., 2003). Thirdly, some recent studies using big animal models have failed to show complete modulation of the cardioprotective effect of ischemic preconditioning by these two drugs (Sanada et al., 2001a; Schwartz et al., 2002).

It is not clear why these discrepancies exist, but some investigators have suggested that opening of sarcolemmal K_{ATP} channels may be more important in the beating hearts to limit stunning, while in *in vitro* experimental conditions opening of mitoK_{ATP} appears to limit cell death (Gross and Peart, 2003). The actual cardioprotective role of these channels still needs to be investigated further.

1.3.2.1.4 The mitogen-activated protein kinase (MAPK)

Mitogen-activated protein kinase (MAPK) are serine-threonine protein kinases that are activated by diverse stimuli ranging from cytokines, growth factors, neurotransmitters, hormones, cellular stress, and cell adherence. They function in a three-tier module comprising of a MAPK kinase kinase, a MAPK kinase and a MAPK. The mammalian MAPK can be subdivided into five families: 42 and 44-kDa extracellular signal-regulated kinase (Erk1/2), p38, the c-Jun NHP_2 terminal kinase (JNK), Erk 3/4 and Erk 5 (Widmann et al., 1999).

The role of Erk1/2 as a potential mediator of IP has been controversial, with the majority of studies supporting its role in the IP's cardioprotection (Hausenloy and Yellon, 2006). In response to preconditioning stimulus, Erk1/2 was found to redistributes to the nucleus, intercalated discs, cytosol and mitochondria. Erk1/2 can phosphorylate and inhibit GSK-3β (Eldar-Finkelman et al., 1995), the consequence of which is inhibition of

mPTP in different settings of cardioprotection (Juhaszova et al., 2004). Thus it is worthy to investigate whether Erk1/2 mediates IP's cardioprotection by inhibiting mPTP opening.

Weinbrenner et al. (Weinbrenner et al., 1997) were the first to report that IP caused p38MAPK activation during ischemia in preconditioned rabbit heart. However, p38MAPK was not activated during ischemia in the control group. Contradictory results were obtained in 1999 by in vivo (Ma et al., 1999) and *in vitro* (Mackay and Mochly-Rosen, 1999) studies suggesting that p38MAPK activation could promote ischemic damage. Interestingly, in the latter study, prolonged ischemia was found to induce biphasic activation of p38MAPK in rat cardiomyocytes, with a transient peak occurring within minutes and followed by a sustained activation after 2h (Mackay and Mochly-Rosen, 1999). Another study performed by Sanada revealed that IP caused a transient but strong activation of p38MAPK. Treatment with SB 203580, a selective p38MAPK inhibitor, during IP blunted the infarct size-limiting effect of IP, while, conversely, the presence of the inhibitor during sustained ischemia partially mimicked the protection of IP (Sanada et al., 2001b). This observation led to the hypothesis that p38MAPK activation has opposing effects; that is, transient activation during IP prevents ischemic injury, while continuous activation during sustained ischemia exacerbates it.

1.3.2.1.5 Phosphoinositide 3 kinase (PI3-K) and Akt

PI3-K, the kinase phosphorylating the plasma membrane lipid phosphatidyl-inositol-4,5-bisphosphate to phosphatidyl-inositol-3,4,5-triphosphate, is implicated in a diverse group of cell functions, including cell growth, cell differentiation, cell survival and intracellular trafficking. Many of these functions are related to its ability to activate Protein Kinase B (PKB, also called Akt) (Cantley, 2002).

In 2000, Tong et al. (Tong et al., 2000) first demonstrated that IP protects the heart by activating the PI3K–Akt pathway and were later supported by several other studies (Mocanu et al., 2002; Yamaura et al., 2003). Inhibition of PI3-K was shown to attenuate IP's cardioprotective effect and also block the phosphorylation of Akt following IP. Akt is known to be able to activate anti-apoptotic pathways (Hausenloy and Yellon, 2004). A recent study by Davidson et al. demonstrated that over-expressing Akt protected cells against oxidative stress by inhibiting mPTP opening (Davidson et al., 2006).

1.3.2.2 Cellular mechanisms of the late phase of IP

The late phase of ischemic preconditioning provides far more prolonged cardioprotection than the early phase (48-72 hours versus 2 to 3 hours), which gives rise to the notion that the late phase may ultimately have greater clinical usefulness.

1.3.2.2.1 Adenosine

The concept that adenosine released during the brief ischemia stimulus triggers the development of delayed protection was first proposed by Baxter et al. (Baxter et al., 1994) and subsequently expanded by the same group (Baxter and Yellon, 1997a; Dana et al., 1998) and others (Auchampach et al., 1999; Takano et al., 1999). Activation of adenosine receptors has been reported to provide delayed protection only against myocardial infarction but not against myocardial stunning or arrhythmias (Maldonado et al., 1997; Auchampach et al., 1999). And such delayed protection can be triggered by activation of either adenosine A1 or A3 receptors (Auchampach et al., 1999; Takano et al., 1999). However, it is not clear whether only one or both of these adenosine receptor subtypes contributes to triggering delayed IP, because 8-psulfophenyl theophylline, the only

adenosine receptor antagonist shown to block the development of late IP (Baxter et al., 1994), is not selective between A1 and A3 receptors. Studies have shown that selective stimulation of adenosine A1 receptors activates p38MAPK-HSP27 pathway via a PKC-dependent mechanism (Dana et al., 2000b) and increases the synthesis of manganese superoxide dismutase (Mn–SOD) (Dana et al., 2000a), while the role of A3 receptors is yet to be established.

1.3.2.2.2 Reactive Oxygen Species (ROS)

ROS includes oxygen ions, free radicals, and peroxides. They are highly reactive due to the presence of unpaired valence shell electrons. While a large burst of ROS results in cell damage, moderate release of ROS can act as an alarm to warn the myocardium to switch to a defensive phenotype. An obligatory role of ROS in the delayed protection induced by IP was first discovered by Sun et al. (Sun et al., 1996). These investigators demonstrated in conscious pigs that the administration of a combination of antioxidants (superoxide dismutase [SOD] plus catalase plus mercaptopropionyl glycine [MPG]) during the initial ischemic challenge prevented the development of late protection against stunning. MPG has also been found to prevent the late protection against infarction, arrhythmias (Yamashita et al., 1998), and coronary endothelial injury (Kaeffer et al., 1997). In contrast, intracoronary infusion of an ROS-generating solution in rabbits elicits a late IP-like response (Takano et al., 1997). These findings provided strong evidence that sublethal oxidative stress is essential to initiate the protection observed in the late phase of IP.

1.3.2.2.3 Nitric Oxide (NO)

Nitric oxide (NO) is an endogenous vessel relaxant that was initially identified as endothelium-derived relaxation factor (EDRF). It is generated from the amino acid L-arginine by various nitric oxide synthase (NOS). There are 3 forms of NOS: endothelial (eNOS), neuronal (nNOS), and inducible (iNOS), each with separate functions. The two isotypes present in the cardiovascular system are eNOS and iNOS.

The first indication that NO triggers late IP was provided by a study in which administration of N^{w}-nitro-L-arginine (L-NA), a nonselective inhibitor of all NOS isoforms, before preconditioning stimulus blocked the development of delayed protection against myocardial stunning (Bolli et al., 1997a). A subsequent study demonstrated that NO is also necessary to trigger delayed protection against myocardial infarction (Qiu et al., 1997). Importantly, exposure to exogenous NO is sufficient to reproduce late protection against both myocardial stunning and infarction that is observed during the late phase of IP (Takano et al., 1998b).

NO plays a dual role in the genesis of late cardioprotection of IP, acting initially as a trigger and subsequently as a mediator. Immediately after preconditioning stimulus, there is an increase in eNOS activity in the myocardium (Xuan et al., 2000). The enhanced biosynthesis of NO by eNOS is important to trigger the development of the delayed cardioprotection, which can be abrogated by non-selective NOS inhibitor but not by relatively selective inhibitors of iNOS applied before the preconditioning stimulus (Bolli et al., 1997b). However, 24 hours after the brief I/R stimulus, iNOS seems to take over and mediate the late protection. Evidence was first provided by two studies in conscious rabbits, in which the delayed protection against both myocardial stunning (Bolli et al., 1997b) and infarction (Takano et al., 1998a) was abrogated by administration of

relatively selective iNOS inhibitors 24h after preconditioning, just before the lethal ischemia. Guo et al. (Guo et al., 1999) later demonstrated that the late phase of IP is associated with upregulation of myocardial iNOS (whereas eNOS remains unchanged) and that targeted disruption of the *iNOS* gene eliminated the delayed infarct-sparing effect. Taken together, the two isoforms of NOS are sequentially involved the late phase of IP, with eNOS generating the NO that initiating the development of IP response on day 1 and iNOS generating the NO that protects against recurrent ischemia on day 2 (Bolli, 2000).

1.3.2.2.4 PKC

In 1995, Baxter first demonstrated the essential role of PKC in the late phase of IP with a study in which the delayed infarct-sparing effects of IP in rabbits were abrogated by pretreatment with the PKC inhibitor chelerythrine (Baxter et al., 1995). Conversely, administration of the PKC activator dioctanoyl-*sn*-glycerol induced cardioprotection 24 hours later (Baxter et al., 1997b). Subsequent studies revealed that IP caused selective translocation of PKCε and PKCη but did not affect the other 8 isoforms expressed in the rabbit heart and did not significantly change total PKC activity (Ping et al., 1999b). In the same model, pretreatment with the PKC inhibitor chelerythrine at doses that have previously been shown to block IP's protection blocked the translocaiton of PKCε (Ping et al., 1999b), whereas the same inhibitor at a 10-fold lower dose shown to only block the translocation of PKCη failed to abrogate the late protection of IP (Qiu et al., 1998). These findings suggest that activation of PKC after ischemic preconditioning is isoform selective and ε seems to be the specific PKC isozyme responsible for the development of delayed protection in this setting. The IP-induced activation of PKCε is likely to be

caused by the generation of NO during the initial ischemic stress according to Ping's study, where they found that such activation was blocked by pretreatment with L-NA (Ping et al., 1999a). In the same study, they also observed that administration of NO donors in the absence of ischemia induced a selective activation of PKCε to an extent comparable to that induced by IP, while coadministration of chelerythrine blocks both the activation of PKCε and the delayed protection elicited by the NO donors (Ping et al., 1999a).

Other Preconditioning studies have implicated PKC-α, PKC-δ, and PKC-ε in the rat heart (Yoshida et al., 1997), and PKC- α in the dog heart (Kitakaze et al., 1997). Interestingly, in an isolated rat heart model, PKC-δ and PKC-ε are demonstrated to play opposing roles in cardioprotection, with activation of the former being detrimental and activation of the latter being protective (Inagaki et al., 2003). In another study using a mouse model, transgenic expression of constitutively-activated PKCε in the heart was shown to be able to recapitulate both the signaling events and the late protection of IP (Ping et al., 2000).

1.3.2.2.5 K_{ATP} channel

Pharmacological studies have provided evidence that opening of K_{ATP} channels is necessary for the delayed infarct-sparing effects induced by ischemic preconditioning (Bernardo et al., 1999; Takano et al., 2000), adenosine A1 and A3 receptor agonists (Takano et al., 1999), and opioid receptor agonists (Fryer et al., 1999). These diverse preconditioning stimuli converge on K_{ATP} channels, suggesting that the activity of these channels may be a common distal effector of delayed protection against cell death. However, the IP-induced late protection against stunning does not appear to require K_{ATP}

channel activity (Takano et al., 1999). The different role of K_{ATP} channels in late IP against stunning versus against infarction provides evidence that different mechanisms underlie these two forms of cardioprotection.

Major issues that remain to be elucidated are the identity of the K_{ATP} channels involved in the late phase of IP (i.e., sarcolemmal versus mitochondrial) and the mechanism whereby their opening confers protection. Given the limitations of the available pharmacological tools, it has been suggested that molecular approaches such as gene targeting and transgenesis will be required to definitively assess the role of mitochondrial versus sarcolemmal K_{ATP} channels (Bolli, 2000).

1.3.2.2.6 Transcription factors

Transcription factors govern the expression of the cardioprotective genes responsible for late IP. The first transcription-regulatory element identified in the late IP signaling mechanisms was nuclear factor-kB (NF-kB) (Xuan et al., 1999), which is known to be a major modulator of iNOS, COX-2, and aldose reductase gene expression. Using conscious rabbits, Xuan and colleagues found that IP induced rapid activation of NF-kB. Inhibition of NF-kB with diethyldithiocarbamate completely abrogated the cardioprotective effects observed 24 hours later. They further demonstrated that the IP-induced activation of NF-kB was blocked by pretreatment with L-NA (NOS inhibitor), MPG (antioxidant), chelerythrine, and LD-A (protein tyrosine kinase [PTK] inhibitor), all given at doses previously shown to block late IP. This finding indicates that the cellular mechanism whereby IP activates NF-kB involves the formation of NO and ROS and the subsequent activation of PKC and PTK-dependent signaling events. Thus, NF-kB appears

to be a common downstream pathway though which multiple signals elicited by ischemic stress (NO, ROS, PKC, and PTKs) act to induce gene expression in the heart.

1.3.2.2.7 Cyclooxygenase-2 (COX-2)

Cyclooxygenase (COX) is the rate-limiting enzyme in prostaglandin (PG) synthesis, catalyzing the conversion of arachidonic acid to PGH_2 (Smith et al., 1996). Two distinct COX isoforms have been characterized so far: COX-1, which is present in most cells and is responsible for constitutive prostanoid formation, and COX-2, which is inducible and becomes abundant in activated macrophages and other cells at sites of inflammation.

Induction of COX-2 was generally thought detrimental. However, the role of COX-2 in cardiovascular system has been found to be beneficial. Recent studies have demonstrated that ischemic preconditioning upregulates the expression and activity of COX-2 in the heart 24h after preconditioning, concomitant with an increase in the myocardial levels of PGE_2, 6-keto-$PGF_{1\alpha}$ (the stable metabolite of PGI_2), and (to a lesser extent) $PGF_{2\alpha}$ (Shinmura et al., 2000). Administration of 2 unrelated COX-2-selective inhibitors (NS-398 and celecoxib) 24 hours after IP abolishes the increase in prostanoids and, at the same time, completely blocks the cardioprotective effects of late IP against both myocardial stunning and infarction. These observations identify COX-2 as a cardioprotective protein and strongly point to PGE_2 and/or PGI_2 as the likely effectors of COX-2–dependent protection. Subsequent studies by the same group further addressed the mechanism underlying the regulation of COX-2 by IP (Shinmura et al., 2002). Using conscious rabbit model, they found that induction of COX-2 protein expression in preconditioned myocardium requires PKC-, Src/Lck PTK-, and NF-κB-dependent

signaling. On the other hand, iNOS-derived NO was required for the activity of newly synthesized COX-2 following IP.

1.3.2.2.8 Heat shock protein (HSP)

Heat shock proteins (HSP) are a group of highly conserved proteins whose expression is increased when the cells are exposed to elevated temperatures or other stress. Each HSP is named according to its molecular weights. The best characterized HSPs, hsp90, hsp70 and hsp65, are induced in response to heat in all organisms studied from bacteria to human.

In 1991, Knowlton et al. first reported the expression of HSP in rabbit after brief ischemia challenge (Knowlton et al., 1991). Marber et al. confirmed this finding (Marber et al., 1993) and further demonstrated in a later study that there is a correlation between the amount of hsp70 induced and the ability to limit infarct size (Marber et al., 1994). This led to the hypothesis that the protection of late IP is mediated by these chaperon proteins. In 1995–1996, several groups successfully generated transgenic mice that over-expressed hsp70 in the heart and other organs (Marber et al., 1995; Plumier et al., 1995; Radford et al., 1996). In all cases, these groups were able to demonstrate that such over-expression of hsp70 protected the heart against ischemia damage using a variety of endpoints such as infarct size, creatine kinase release, recovery of high energy phosphate stores, and correction of metabolic acidosis.

Recently, another member of HSP, hsp27 has been suggested to participate in the late protection of IP as a downstream target of p38MAPK (Nakano et al., 2000; Huot et al., 1997; Hedges et al., 1999). The translocation of hsp27 from cytosol to myofibril or nucleus may prevent actin fragmentation (Huot et al., 1996) or microtubule degradation

(Bluhm et al., 1998). On the other hand, hsp27 prevents the interaction of Apaf-1 with procaspase-9 through binding to cytochrome c (Garrido et al., 1999). Both of these actions can ameliorate or delay ischemic cell death.

1.3.3 Pharmacological preconditioning

Despite the powerful protection provided by ischemic preconditioning, the clinical implement of this approach faces practical problems. Because brief ischemia challenge must precede lethal ischemia to achieve cardioprotection, induction of preconditioning ischemia doesn't serve as a realistic therapeutic strategy for patients with ischemic heart disease. However, if the signaling mechanisms underlying IP can be determined, more simple and effective therapeutic intervention can be developed accordingly. Considerable progress has been made to understand the signaling mechanism and to identify the substances that are capable of duplicating the cardioprotection induced by IP. These substances can be divided into two categories: naturally occurring but often noxious agents, e.g. endotoxin (Brown et al., 1989), interleukin-1 (Brown et al., 1990), tumor necrosis factor-α (Brown et al., 1992), leukemia inhibitory factor (Nelson et al., 1995), ROS (Sun et al., 1996), and clinically applicable drugs including K_{ATP} channel opener (Sato et al., 2000), NO releasing agents (Takano et al., 1998b), adenosine receptor agonists (Baxter et al., 1994), and opioid receptor agonists (Fryer et al., 1999). Most of these forms of preconditioning have been shown to protect against lethal ischemia/reperfusion injury, and some have been demonstrated to protect against reversible postischemic dysfunction (Sun et al., 1996) and endothelial dysfunction (Kaeffer et al., 1997). Further efforts should be directed to promote more clinical studies based on the evidence from experimental studies in larger animals.

1.4 Hydrogen Sulfide (H_2S)

For many decades, H_2S has been receiving attention as a toxic gas and environmental hazard. Its physiological importance was not recognized until the recent finding that H_2S occurs naturally in mammalians. This led to the discovery of H_2S as the third gasotransmitter after NO and CO. In the following content, recent works on H_2S will be reviewed with an emphasis on its biological effects and its roles in different diseases.

1.4.1 Physical and chemical properties of H_2S

Hydrogen sulfide (H_2S) is a colorless, flammable and water soluble gas with smell of rotten eggs. The detectable level of this gas by human olfaction is 400 folds lower than its toxic level. In an aqueous solution, H_2S is a weak acid which dissociates in the following reaction: $H_2S \Leftrightarrow H^+ + HS^- \Leftrightarrow 2H^+ + S^{2-}$. According to the Henderson–Hasselbach equation, it will form approximately 18.5% H_2S and 81.5% hydrosulfide anion (HS) in a physiological solution (pH 7.4, 37°C) (Dombkowski et al., 2004). H_2S is a highly lipophilic molecule, which enables it to freely penetrate cells of all types and become biologically active.

1.4.2 Endogenous generation and metabolism of H_2S

It is known that certain bacteria and archae can produce H_2S. Recently, mammalian cells have also been found to be able to generate and metabolize H_2S. For example, the H_2S concentration in rat serum was reported to be around 46μM (Zhao et al., 2001). A higher level of H_2S was detected in the brain and reported to be 50~160 μM (Abe and Kimura, 1996). However, such remarkably high concentrations of H_2S have recently been questioned by some reviews (Li and Moore, 2008; Szabó, 2007) and a group of

researchers (Whitfield et al., 2008) who failed to detect H_2S at micromolar level in the blood of a variety of animals. Indeed, the evanescent and reactive nature of this gas makes it difficult to accurately measure its concentration in an aqueous solution. A direct, reliable and stable means of detection needs to be developed before a conclusion can be drawn.

Despite the discrepancy on the exact concentration of circulating sulfide, two pyridoxal-5-phosphate-dependent enzymes have been identified as the endogenous synthases of H_2S, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE). Both of them use L-cysteine as the main substrate and collectively are responsible for the majority of H_2S production in the body (Stipanuk and Beck, 1982; Erickson et al., 1990; Bukovska et al., 1994). The expression of CBS and CSE has been detected in a board variety of cell types, including those from liver, kidney, heart, brain, skin fibroblastes, and lymphocytes (Wang, 2002). In some tissues, both CBS and CSE contribute to the local generation of H_2S, whereas in others, one enzyme predominates. CBS is the predominant H_2S synthase in the brain and nervous system and is highly expressed in liver and kidney, while CSE is primarily expressed in the heart, liver, vascular and non-vascular muscles.

H_2S *in vivo* is metabolized by oxidation in mitochondria before it is excreted through urine as free or conjugated sulfate (Beauchamp et al., 1984). Nevertheless the mechanism of the conversion of sulfide to sulfate or thiosulfate is poorly understood. The role of a sulfide oxidase and/ or glutathione has been proposed. A less important metabolic pathway involves methylation of sulfide by cytosolic *S*-methyltransferase. It is worthy of note that H_2S can also be scavenged by methemoglobin (Beauchamp et al., 1984) or

metallo- or disulfide-containing molecules such as oxidized glutathione (Smith and Abbanat, 1966). Hemoglobin may act as a common sink for CO, NO and H_2S (Wang, 1998). It interacts with H_2S by forming green sulfhemoglobin (Arp et al., 1987). Caution should be raised if this sink is saturated by one of the three gases; then its ability to bind other gases would be reduced (Searcy and Lee, 1998).

1.4.3 Biological role of H_2S

1.4.3.1 H_2S and the central nervous system (CNS)

The presence of considerable amounts of H_2S and its synthase CBS in the brains of several species including humans suggested a role for this gas in CNS function (Kimura, 2002). H_2S has been shown to facilitate induction of hippocampal long-term potentiation by increasing the sensitivity of NMDA receptors (Kimura, 2000). Interaction of H_2S and NMDA receptors possibly involves cAMP-dependent protein kinase pathway, since in the same study NaHS increases cAMP levels in neuronal and glial cell lines and primary neuron cultures. In addition, H_2S was also reported to induce protection against glutamate-mediated toxicity in cortical neurons (Kimura et al., 2006) and mouse hippocampal cell line (Kimura and Kimura, 2004), perhaps by multiple mechanisms including activation of K_{ATP} and Cl^- channels and elevation of intracellular glutathione (Kimura et al., 2006).

Deranged biosynthesis of H_2S has been found to be associated with central nervous system diseases, such as stroke (Qu et al., 2006), Down syndrome (Kamoun et al., 2003), and perhaps also Alzheimer's disease (Eto et al., 2002). In the rat model of stroke (Qu et al., 2006), middle cerebral artery occlusion caused an increase in H_2S level in the lesioned cortex as well as an increase in the H_2S synthesizing activity. In keeping with this,

administration of a sulfide donor significantly increased the infarct volume. In subjects with Down syndrome, urinary thiosulfate (a metabolite of H_2S) and erythrocyte sulfhemoglobin levels were both significantly increased compared with diet-matched controls (Kamoun et al., 2003). Further studies are warranted to determine whether the abnormity of H_2S level is a cause or simply a consequence of these diseases.

1.4.3.2 H_2S and inflammation

Extensive studies have recently been conducted to define the role of H_2S in various inflammatory diseases. At micromolar concentrations, H_2S can induce an upregulation of anti-inflammatory and cytoprotective genes including haem oxygenase-1 in pulmonary smooth muscle cells *in vivo* (Qingyou et al., 2004) and in macrophages *in vitro* (Oh et al., 2006). H_2S also reduces LPS-stimulated TNF-α and NO formation in cultured microglial cells (Hu et al., 2006). In animal models of inflammation, administration of H_2S donor has been effective in reducing carrageenan-induced paw edema and air pouch-induced leukocyte infiltration (Zanardo et al., 2006), the commonly-used systems to test the anti-inflammatory effects of experimental compounds. The protective effect of H_2S was attenuated by pretreatment with glibenclamide, suggesting the involvement of K_{ATP} channels. Several other studies demonstrated that chemically linking an H_2S-donor species to known anti-inflammatory drugs can improve the therapeutic profile of the compound. Using a rat model of endotoxin-induced inflammation, Li and colleagues reported that a sulfide-releasing compound, S-diclofenac, enhanced the anti-inflammatory effect of the parent molecule and exhibited less gastric toxicity (Li et al., 2007). Similarly, in the study by Distrutti et al., the H_2S-releasing derivative of mesalamine demonstrated superior anti-inflammatory and antinociceptive efficacy compared with the base

mesalamine molecule in the model of postinflammation hypersensitivity (Distrutti et al., 2006).

However, it is paradoxical that an upregulation in H_2S-synthesizing activity or plasma H_2S level was observed in a large body of studies using different inflammation models. These include carrageenan-induced paw oedema in rats (Bhatia et al., 2005a), a mouse model of pancreatitis (Bhatia et al., 2005b), rodent model with endotoxic shock (Collin et al., 2005; Li et al., 2005), and a polymicrobial sepsis model in mice with cecal ligation and puncture (Zhang et al., 2006). Pharmacological inhibitor of H_2S biosynthesis, DL-propargylglycine (PAG) (Marcotte and Walsh, 1975), was used in some of these studies and shown to be able to attenuate the inflammatory responses. In a rat model of endotoxemia, PAG prevented the increases in the serum levels of liver and pancreas injury markers and reduced the tissue content of myeloperoxidase (Collin et al., 2005). In a model of cecal ligation and puncture, PAG treatment reduced tissue neutrophil infiltration and improved liver and lung histology (Zhang et al., 2006). In a carrageenan-induced inflammation model in the rat, PAG treatment dose-dependently reduced paw edema and neutrophils infiltration (Bhatia et al., 2005a).

Interestingly, both inhibitor and donor of H_2S were shown to exert beneficial effects in the same experimental model of disease, for instance, in the carrageenan paw edema model (Bhatia et al., 2005a; Zanardo et al., 2006). While one study demonstrates anti-inflammatory effects of H_2S (Zanardo et al., 2006), another one argues for a pro-inflammatory role of H_2S in the same model (Bhatia et al., 2005a). A reasonable explanation for these conflicting results is that endogenous sulfide at low and high local concentrations exert opposing effects, with low concentration preventing and high

concentration promoting inflammation. A similar paradox has been previously noted with inhibitors versus donors of NO — both of them being effective in the carrageenan paw edema models (Handy and Moore, 1998; Fernandes et al., 2002). Clearly, there is an exquisite balance and a complex regulation of pathophysiological responses by endogenous and exogenous gasotransmitters (Szabó, 2007).

1.4.3.3 H_2S and cardiovascular system

It was conventionally held that H_2S interfered with cardiovascular function as a result of the secondary anoxia rather than a direct action of the gas on cardiac myocytes or vascular smooth muscle cells (SMCs) (Reiffenstein et al., 1992). However, this view has been overturned by the finding of noticeable amount of H_2S and its synthase CSE in the cardiovascular system.

As early as in 1997, expression of CSE and endogenous production of H_2S have been detected in rat portal vein and thoracic aorta (Hosoki et al., 1997). A more recent study by Zhao et al. revealed that CSE is the only H_2S-generating enzyme in rat mesenteric artery and other vascular tissues, with expression levels of CSE mRNA ranked in an order of pulmonary artery > aorta > tail artery > mesenteric artery (Zhao et al., 2001). On the other hand, Chen et al. found no activity or expression of CBS in human atrium and ventricle tissues (Chen et al., 1999). The activity and/or expression of CBS were also lacking in human internal mammary arteries, saphenous veins, coronary arteries, or aortic arteries (Chen et al., 1999; Bao et al., 1998). Thus, only CSE appears to be responsible for the generation of H_2S in cardiovascular tissues.

The effect of H_2S on vascular systems has recently been investigated in several in-depth studies. H_2S at physiologically relevant concentrations induces relaxation in portal

vein (Hosoki et al., 1997), aorta (Zhao et al., 2001), and mesenteric artery beds of rats (Cheng et al., 2004). In one study by Zhao et al. (Zhao et al., 2001), an intravenous bolus injection of H_2S at 2.8 and 14 μmol/kg body weight provoked a transient decrease in mean arterial blood pressure of anaesthetized rats. At the tissue level, H_2S induced a concentration-dependent relaxation of the phenylephrine (PHE)-precontracted rat aortic tissues (IC_{50}, 125 μM). The investigators also provided significant insight into the mechanism of the H_2S-induced vasorelaxant effect (Zhao et al., 2001). They found that when isolated rat aortic tissues were precontracted with 20 or 100 mM KCl, the maximum vascular relaxation induced by H_2S was 90% and 19%, respectively. This difference in relaxation potency of H_2S may represent the portion of relaxation possibly mediated by potassium conductance. Furthermore, the effect of H_2S on aortic tone was only antagonized by the blocker of K_{ATP} channel, glibenclamide, but not blockers of other types of potassium channels, indicating that the vasorelaxant effect of H_2S was K_{ATP}-dependent. Meanwhile, they demonstrated that H_2S directly increased K_{ATP} channel currents and hyperpolarized membrane in isolated SMCs. Taken together, all these lines of evidence point to that H_2S is an important endogenous vasorelaxant factor and gaseous opener of K_{ATP} channels in vascular SMCs.

In spite of the detection of CSE in myocardial tissues, the effect of H_2S on heart was relatively less known. Geng et al. observed a negative inotropic effect of H_2S in both *in vitro* and *in vivo* experiments, and the effect could partly be blocked by glibenclamide (Geng et al., 2004). As discussed by the investigators, in addition to direct effect of H_2S on myocardium, the cardiac contraction could also be affected by its peripheral vascular effect, because H_2S dilated arteries and veins, reducing central venous pressure, which

could result in a decrease of the venous return and reduction of cardiac pre- and post-loads. Although this study is suggestive of a role for H_2S in regulating cardiac function, the significance of these observations should further be determined in pathological settings.

1.4.3.4 Other effects of H_2S

Sulfide may also have therapeutic potential in the areas of angiogenesis and wound healing. Studies have demonstrated that H_2S stimulates endothelial cell growth, adhesion, migration and promotes scratched wound healing in vitro, probably through a PI3-K/Akt pathway (Cai et al., 2007). Intraperitoneal injection of NaHS for 7 days significant increases neovascularization in rats. Other studies showed that sulfide donors promote gastric ulcer healing in rodent models (Wallace et al., 2007; Yonezawa et al., 2007), but the effect did not appear to depend on NO synthesis or K_{ATP} opening.

1.4.3.5 Interaction of H_2S and other gasotransmitters

H_2S is the third gasotransmitters together with NO and CO. It is not surprising that these mediators interact with one another in regulating cell functions, cardiovascular responses and inflammatory/immune functions. Published data have shown that the endogenous production of H_2S from rat aortic tissues is enhanced by NO donor treatment (Zhao et al., 2001). The NO donor also enhances the expression level of CSE in cultured vascular SMCs. Hosoki et al. (Hosoki et al., 1997) observed that low concentrations of H_2S (30μM) markedly enhanced the vasorelaxant effect of the NO donor sodium nitroprusside (SNP). On the contrary, pretreating aortic tissues with a higher dose of H_2S (60μM) inhibited the vasorelaxant effect of SNP. Apparently, there is a complex crosstalk between H_2S and NO, where direct action on synthases may be involved.

Non-vascular aspects of NO–H_2S interactions have also been identified: it seems that NO-related effects contribute to the cytoprotective and antinociceptive effects of sulfide (Fiorucci et al., 2006). In macrophages, H_2S inhibits the expression of iNOS but upregulates the expression of heme oxygenase-1 (Oh et al., 2006). H_2S also acts as a scavenger and neutralizer of peroxynitrite (Whiteman et al., 2004), a key player in the cytotoxic effects of NO.

CO may be another factor capable of interacting with H_2S and NO. As noted above, H_2S can also upregulate HO1, and can therefore induce a delayed production of CO. Given that all three bind avidly to hemoglobin, saturation of hemoglobin with one could lead to enhanced plasma levels and biological effects of the others (Wang, 1998). Indeed, saturation of erythrocytes with CO results in elevated plasma H_2S levels (Searcy and Lee, 1998). Additional studies are needed to examine the various interplays between the three gasotransmitters in health and disease, and to identify areas in which pharmacological modulation of these agents (alone or in combination) may provide therapeutic benefit (Szabó, 2007).

1.5 Objectives and significance of the present study

Studies have suggested that H_2S plays a part in regulating heart contractility under physiological situation (Geng et al., 2004). However, the effect of H_2S on the heart under pathological situation, such as ischemia, is still unknown. To date, most of the known effects of H_2S are mediated through an action on K_{ATP} channels. Considering that opening of K_{ATP} channels is an essential event for development of ischemic preconditioning-induced cardioprotection and this protection could be mimicked by pharmacological opener of K_{ATP} channels, it merits study to investigate whether H_2S

could exert a similar beneficial effect through opening K_{ATP} channels on a heart undergoing ischemic challenge.

Therefore the objective of current study was to delineate the role of H_2S in the cardioprotection against ischemia injury. To be specific, 1) endogenous H_2S levels were assessed in both normal and ischemia-insulted myocardial tissues to examine whether endogenous H_2S production altered during ischemia insult; 2) inhibitors of H_2S biosynthesis were employed to investigate whether endogenous H_2S contributed to the cardioprotection induced by ischemic preconditioning; 3) a sulfide donor was applied in an attempt to observe whether exogenous H_2S could increase the resistance of myocardial tissues to ischemic attacks. Both *in vitro* and *in vivo* models were used to verify these issues at cellular level and the whole animal level. At the cellular level, isolated cardiomyocyte model was adopted due to its advantage in studying signaling mechanisms without confounding factors from the whole circulation. At whole animal level, a rat model of myocardial infarction was employed, as rats share considerable similarity on the cardiovascular system with humans.

Accordingly, the findings from current study were expected to contribute to the knowledge in three areas: pathophysiology of cardiac ischemia, mechanisms of ischemic preconditioning-induced cardioprotection, and the biological profile of H_2S. The results not only revealed a novel mechanism for ischemic preconditioning-induced cardioprotection, but also identified a simple and effective intervention approach for ischemic heart diseases. The results from *in vivo* experiments also provided valuable information for the translation from the bench to the bedside.

Chapter 2 Endogenous H_2S mediates the cardioprotection induced by ischemic preconditioning in rat cardiomyocytes

2.1 Introduction

Hydrogen sulfide (H_2S) represents the most recently identified endogenous gaseous messenger (Wang, 2002). The detection of plentiful H_2S synthase CSE in the heart (Geng et al., 2004) suggests that endogenous H_2S production is necessary for a well-functioned heart. However, it is not clear how the endogenous H_2S level is associated with the heart's condition. Thus we investigated whether there is a difference in endogenous H_2S production between healthy cardiomyocytes and myocytes undergoing ischemia challenge.

Ischemic preconditioning (IP) refers to the phenomenon that previous exposure to brief sublethal ischemia provides protection on the heart against subsequent severe ischemia insults (Murry et al., 1986). It is a powerful natural cardioprotective mechanism. A pivotal signaling event during IP is the opening of K_{ATP} channels (Sanada et al., 2004). The ability of H_2S to open K_{ATP} channels in smooth muscle cells (Zhao et al., 2001) prompted us to investigate whether endogenous H_2S also plays a part in the IP-induced cardioprotection.

2.2 Materials and methods

2.2.1 Isolation of adult rat cardiomyocytes

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

heart 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 (500 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.

2.2.2 Simulation of ischemia and ischemia preconditioning

In the present study, we simulated ischemia with a pH 6.6 glucose-free Kreb's buffer containing 10 mM 2-deoxy-D-glucose (2-DOG), an inhibitor of glycolysis (Macianskiene et al. 2001), and 10 mM sodium dithionite ($Na_2S_2O_4$), an oxygen scavenger (Otter and Austin, 2000). This simulated ischemia solution produces a mixture of effects including metabolic inhibition, anoxia, and acidosis. When the solution was used for preconditioning purpose, the dose of 2-DOG and $Na_2S_2O_4$ were halved to generate a milder ischemic condition.

2.2.3 Experimental protocol

In the present study, we adopted two well-established preconditioning models to observe respectively the first and second phase of IP's cardioprotection. For the induction of early phase of IP (Ho et al., 2002), cardiomyocytes were subjected to three cycle of brief ischemia and reperfusion, with each cycle composed of 3 min of preconditioning ischemia and 5 min of reperfusion with normal Dulbecco's modified Eagle's medium (DMEM) (shown in Fig 2-2A). Lethal ischemia was initiated subsequently and lasted for 9 min, followed by reperfusion for 10 min. For the induction of late phase of IP (Nayeem et al., 1997; Wu et al., 1999), cells were subjected to a single episode of 30 min of preconditioning ischemia. Lethal ischemia was initiated 20 hours later and followed by 10 min of reperfusion (shown in Fig. 2-4A). In each experiment, cells were divided into four groups: vehicle-treated group (VP), IP-treated group (IP), and either DL-propargylglycine (PAG) or β-cyano-L-alanine (BCA) plus IP-treated groups (PAG+IP or BCA+IP). In the latter two groups, 2×10^{-3} mol/L PAG or 2×10^{-3} mol/L BCA were applied during IP. Cells in VP group were incubated with DMEM during preconditioning ischemia.

2.2.4 Measurement of H_2S concentration

The culture media of cardiomyocytes were collected for measurement of endogenous H_2S production. 75 μL media from each sample was added into an Ependorff tube that already contained 450 μL deionised water and 250 μL zinc acetate (1% w/v). Then *N*, *N*-dimethyl-*p*-phenylenediamine sulphate (20 μM in 7.2 mol/L HCl, 133 μl) and $FeCl_3$ (30 μM in 1.2 mol/L HCl, 133 μL) were added in sequence for color development at room temperature. After 10 min, trichloroacetic acid (10% w/v, 250 μL) was added to

precipitate any protein that might be present in the media. The tubes were then centrifuged (10,000 × g) for 3 min and 300 µl aliquots from the resulting supernatants were transferred into a 96-well plate. Absorbance was determined at 670 nm using a 96 well microplate reader (Tecan Systems Inc., U.S.A.).

2.2.5 Assessment of cell viability and morphology

Trypan blue exclusion was used as an index of myocyte viability (Zhou et al., 1996; Hiebert and Ping, 1997). After cells were incubated with 0.4% (w/v) trypan blue dye for 3 min, living cells were unstained and termed nonblue cells. Nonblue cells/total cells were determined in a hemocytometer chamber using a light microscope (10x magnification). Cell morphology was also assessed by microscopic examination (Armstrong and Ganote, 1994; Zhou et al., 1996). Percentage of rod-shaped (length/width ratio, >3:1) cells were determined as an indicator of the percentage of healthy cardiomyocytes. 200-500 cells in each of 5-7 cultures were tested for each group.

2.2.6 Assessment of cellular injury

Lactate dehydrogenase (LDH) release was used as an index of cellular injury (Nayeem et al., 1997). The activity in the cultured medium represents LDH release from the cultured ventricular myocytes. Both culture medium and cell lysates (prepared with lysis buffer containing 1% triton-X100) were collected for determination of LDH activity. LDH assay was performed using a commercially available kit (Sigma). The assay was based on the reduction of NAD catalyzed by LDH. The reduced NAD (NADH) was utilized in the stoichiometric conversion of a tetrazolium dye. The absorbance at a wave length of 490nm was measured spectrophotometrically with a microplate reader (Tecon Systems Inc. U.S.A.). The background absorbance at 690nm was subtracted from the absorbance

at 490nm. The results were presented as LDH released into the medium in terms of percentage of the total LDH activity (medium + cell lysate) and normalized to 100% for VP group.

2.2.7 Measurement of intracellular Ca^{2+} ($[Ca^{2+}]_i$)

Ventricular myocytes were incubated with fura-2/AM (4×10^{-6} mol/L) (25 min) in Tyrode's solution supplemented with 1.25×10^{-3} mol/L $CaCl_2$. The unincorporated dye was removed by washing the cells twice with fresh incubation solution. Loaded cells were kept at room temperature (24 °C-26 °C) for 30 min to allow the fura-2/AM in the cytosol to de-esterify.

Loaded ventricular myocytes were then transferred to the stage of an inverted microscope (Nikon) in a superfusion chamber at room temperature. The inverted microscope was coupled with a dual-wavelength excitation spectrofluorometer (Intracellular imaging Inc, USA). Myocytes were perfused with Krebs' bicarbonate buffer (KB buffer, mmol/L; 117 NaCl, 5 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 1.25 $CaCl_2$, 25 $NaHCO_3$, 11 glucose, with 1% w/v dialyzed BSA) and gassed with 95% O_2/5% CO_2. The myocytes selected for the study were rod-shaped with clear striations. These cells exhibited synchronous contraction (twitch) in response to suprathreshold (4 ms, 0.2 Hz) stimuli delivered by a stimulator (Grass S88) via two platinum field-stimulation electrodes immersed in the bathing fluid. Fluorescent signals obtained at 340 nm (F340) and at 380 nm (F380) excitation wavelengths were stored in a computer for data processing and analysis. The F340/F380 ratio was used to indicate $[Ca^{2+}]_i$ level in the myocytes.

2.2.8 Statistical Analysis.

Values presented are mean ± SEM. One-way ANOVA was used with a post hoc (Bonferroni) test to determine the difference between groups. The significance level was set at $p < 0.05$.

2.2.9 Drugs and chemicals

Type 1 collagenase, protease XIV, 2-DOG, PAG, BCA, *N*,*N*-dimethyl-*p*-phenylenediamine sulfate, $FeCl_3$, and trypan blue dye were purchased from Sigma-Aldrich (St. Louis, MO). Fura-2 was purchased from Invitrogen (Carlsbad, CA). All chemicals were dissolved in distilled water except fura-2/AM, which was dissolved in DMSO at a final concentration < 0.1% (w/v)

2.3 Results

2.3.1Endogenous H_2S production in rat cardiomyocytes was suppressed during ischemia and partly restored by IP

To investigate whether endogenous H_2S level is altered during ischemia, H_2S concentration in cell culture medium after 9 min of ischemia was determined. The experimental procedures are shown in Fig. 2-1A and described under *Materials and Methods*. As shown in Fig. 2-1B, 9 min of ischemia significantly decreased endogenous H_2S level to 23.7 ± 6.9% of that in VP group ($n = 10$, $p < 0.001$). Interestingly, preconditioning with three cycles of ischemia significantly attenuated the inhibitory effect of ischemia on H_2S production (IP, 49.6 ± 9.5%, $n = 5$, $p < 0.05$ versus ischemia group). These data suggest that IP may protect the heart against ischemia at least partly by enhancing the endogenous production of H_2S.

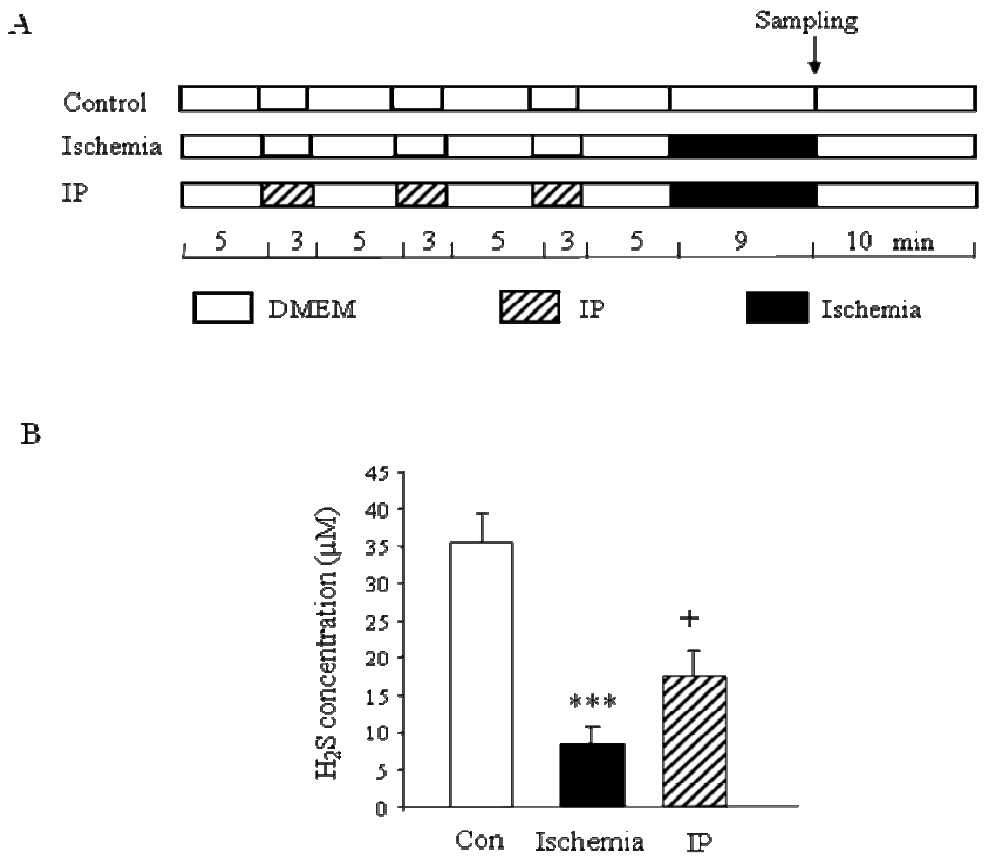

Figure 2-1 Effects of ischemia and IP on endogenous H_2S production. (A) Experimental design. (B)Endogenous H_2S production in normal rat cardiomyocytes (Con), cardiomyocytes undergoing ischemia (Ischemia), and cardiomyocytes subjected to IP (IP). Mean ± S.E.M.; n = 5 to 10. ***, $p < 0.001$ versus Control; +, $p < 0.05$ versus ischemia.

2.3.2 Early cardioprotection induced by IP was blocked by CSE inhibitors

To determine whether endogenous H_2S plays a part in the IP-induced early cardioprotection, PAG and BCA were used as inhibitors of endogenous H_2S synthase and applied according to the protocol shown in Fig. 2-2A. We assessed the protective effects of 3 episodes of brief ischemia and reperfusion on cell viability and cell morphology in the presence and absence of CSE inhibitors. After 9 min of ischemia followed by 10min of reperfusion, the percentage of nonblue cells and percentage of rod-shaped cells in IP

group were significantly higher than those in vehicle-preconditioned (VP) group (Fig. 2-2 B & C). The presence of 2 x 10^{-3} mol/L PAG or 2 x 10^{-3} mol/L BCA during IP reversed the cardioprotection on cell viability and morphology, while neither PAG nor BCA alone affected cell viability (control, 69.2 ± 3.2%; PAG, 63.4 ± 2.4%; BCA, 65.1 ± 1.7%, all n = 5) or morphology (control, 62.8 ± 1.2%; PAG, 60.3 ± 1.8%; BCA, 59.2 ± 1.2%, all n = 5).

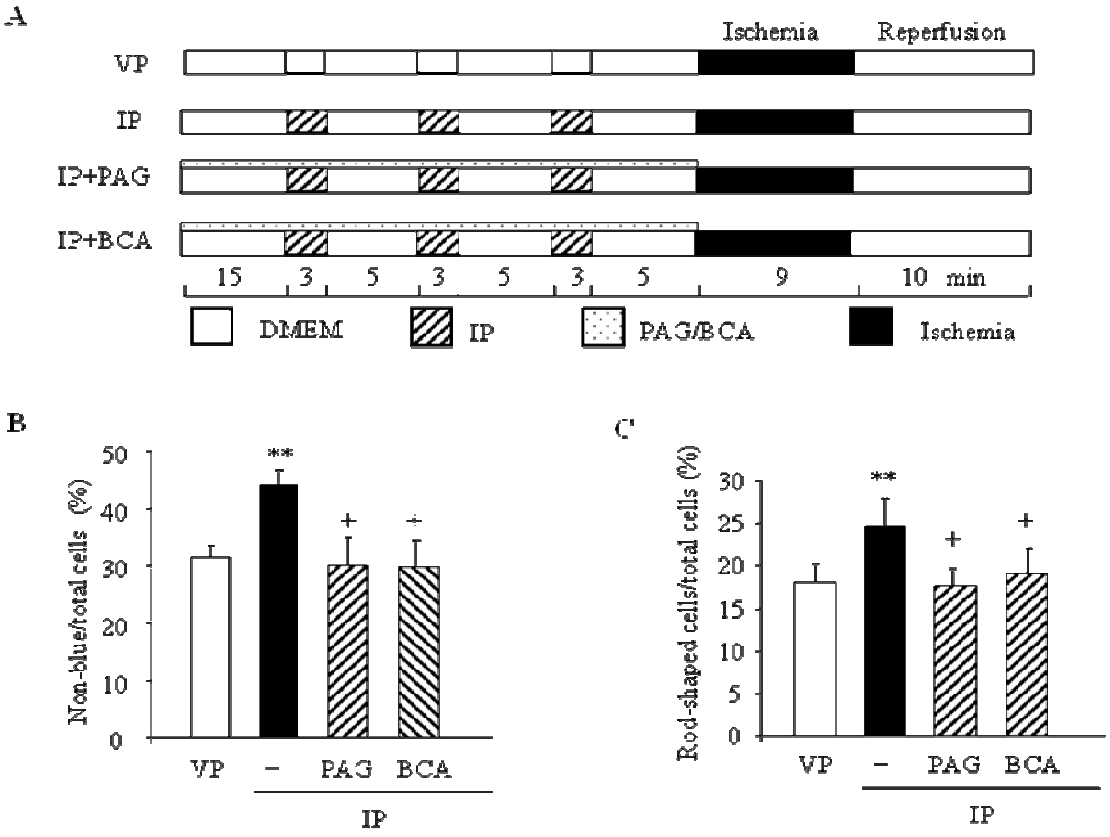

Figure 2-2 Effects of early IP on cell viability and cell morphology in the presence and absence of CSE inhibitors. (A) Experimental protocol. (B) Cell viability at 10 min into reperfusion in VP, IP, IP+PAG, and IP+BCA. (C) Percentage of rod-shaped cells at 10 min into reperfusion in VP, IP, IP+PAG, and IP+BCA. Mean ± S.E.M., n = 6 to 18 cultures of 500 cells each. **, $p < 0.01$ versus VP; +, $p < 0.05$ versus IP.

Electrical stimulation mimics the arrival of an action potential generated from the sino-atrial node of the heart, triggering the same cascade of events i.e.-membrane depolarization, influx of Ca^{2+}, Ca^{2+} release from the SR and finally muscle contraction. We observed the recovery of electrically-induced $[Ca^{2+}]_i$ transients ($E[Ca^{2+}]_i$) during ischemia and reperfusion to investigate whether inhibition of endogenous H_2S formation would affect the cardioprotection of IP on cell function. As shown in Fig. 2-3A, the amplitude of $E[Ca^{2+}]_i$ in VP group following ischemia and reperfusion was reduced to 20.2 ± 3.8% of its initial amplitude. IP remarkably attenuated the wane of $E[Ca^{2+}]_i$ during ischemia and reperfusion and $[Ca^{2+}]_i$ transients in IP group was able to recover to 70.0 ± 3.5% of its initial amplitude. Both PAG and BCA reversed this beneficial effect of IP (PAG, 34.8 ± 3.6%, n = 19; BCA, 37.8 ± 4.6%; n = 10, $p < 0.001$; Fig. 2-3B), while they did not produce significant effect on $E[Ca^{2+}]_i$ when applied alone. These data suggest that endogenous H_2S mediated the early phase of cardioprotection caused by IP on both cell viability and cell function.

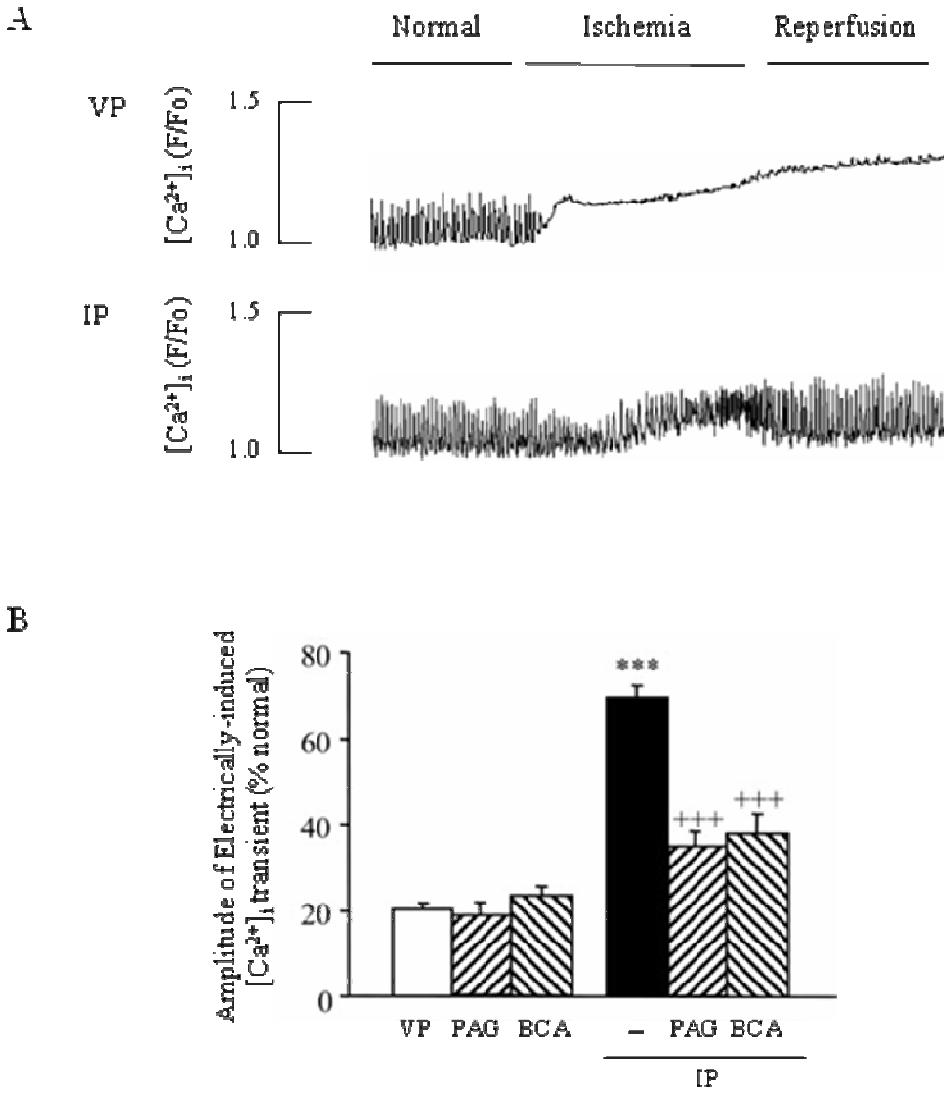

Figure 2-3 Effects of early IP on recovery of electrically-induced $[Ca^{2+}]_i$ transients ($E[Ca^{2+}]_i$) in the presence and absence of CSE inhibitors. Experimental procedures used were the same as those in Fig. 2-2A. (A) Representative tracings of $E[Ca^{2+}]_i$ in VP and IP groups during ischemia and reperfusion. (B) Amplitude of $E[Ca^{2+}]_i$ transients at 5 min of reperfusion. Mean ± S.E.M., n = 10 to 26; ***, $p < 0.001$ versus VP; +++, $p < 0.001$ versus IP.

2.3.3 Late cardioprotection induced by IP was blocked by CSE inhibitors

This series of experiments was designed to determine whether endogenous H_2S is involved in the late phase of cardioprotection induced by IP. Experimental procedures were shown in Fig. 2-4A. Rat cardiomyocytes were subjected to I/R injury 20 hours after preconditioning ischemia. IP significantly increased the percentage of the non-blue cells per total number of cells at 10 min into reperfusion (31.8 ± 3.0%, n = 10, $P < 0.01$) as compared with that found in VP group (20.8 ± 1.7%, $n = 13$). Pretreatment with PAG (2×10^{-3}) mol/L or BCA (2×10^{-3} mol/L) blocked the cardioprotective effect of IP (Fig. 2-4B).

To further substantiate the role of endogenous H_2S, we determined cellular injury using LDH release as an index. As shown in Fig. 2-4C, IP significantly decreased cellular injury caused by severe ischemia insults. Pretreatment with PAG or BCA blocked this effect (Fig. 2-4C).

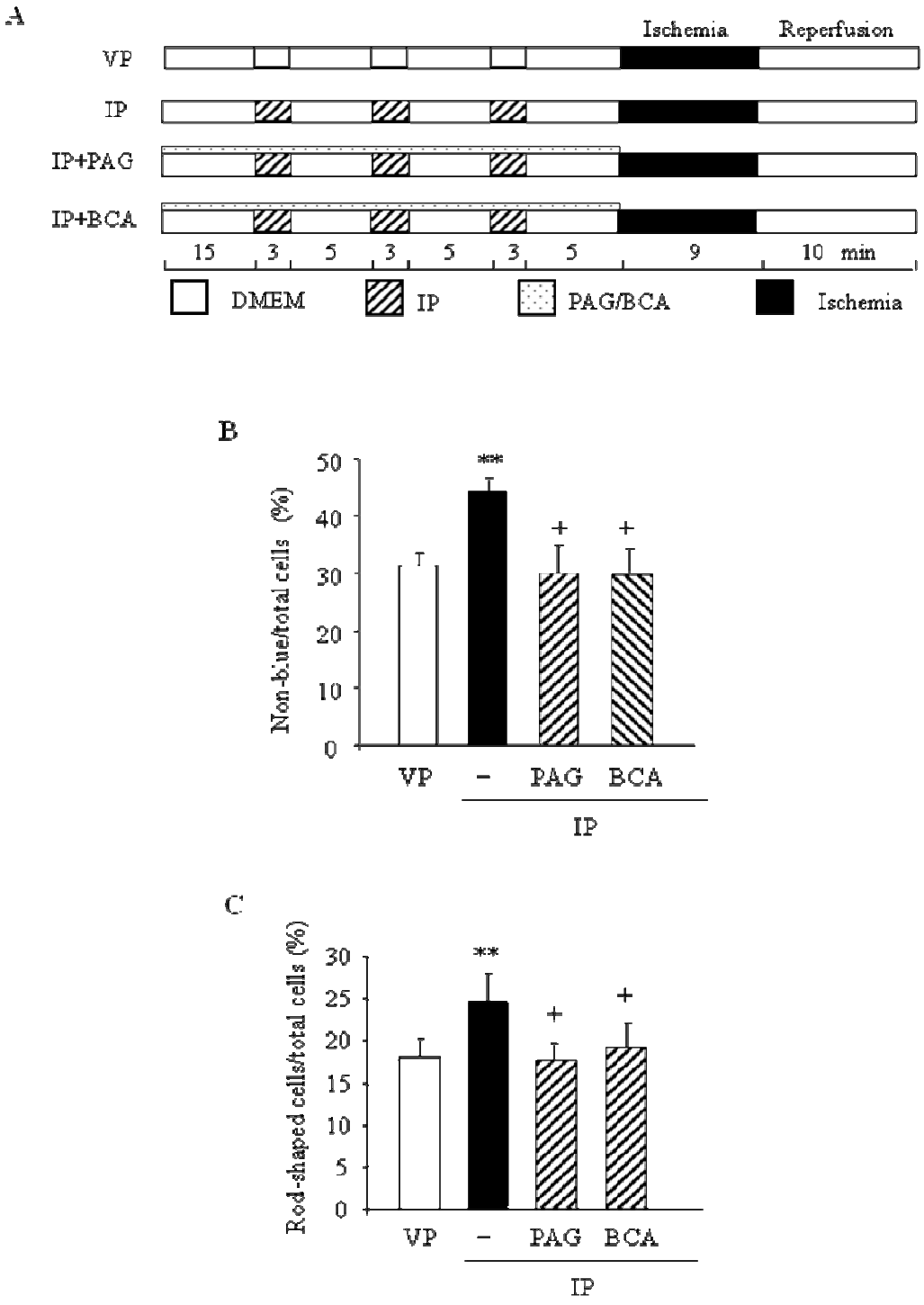

Figure 2-4 Effects of late IP on cell viability and morphology in the presence and absence of CSE inhibitors. (A) Experimental protocol. (B) Cell viability at 10 min into reperfusion in VP, IP, IP+PAG, and IP+BCA. (C) Percentage of rod-shaped cells at 10 min into reperfusion in VP, IP, IP+PAG, and IP+BCA. Mean ± S.E.M., n = 6 to 18 cultures of 500 cells each. **, $p < 0.01$ versus VP; +, $p < 0.05$, ++, $p < 0.01$ versus IP.

The electrically-induced $[Ca^{2+}]_i$ transients were also traced during reperfusion to examine the functional status of the cardiomyocytes. IP significantly improved the recovery of $E[Ca^{2+}]_i$ which was compromised during ischemia (Fig. 2-5 VP & IP). Again, this beneficial effect was reversed by either of the H_2S synthesis inhibitors, PAG (2×10^{-3} mol/L) or BCA (2×10^{-3} mol/L) (Fig. 2-5). Taken together, these data suggest that endogenous H_2S was also involved in the late cardioprotection induced by IP.

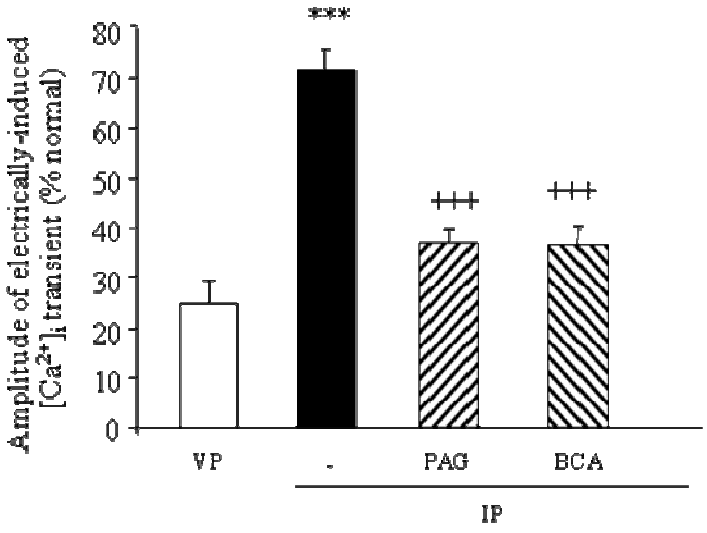

Figure 2-5 Effects of late IP on recovery of electrically-induced $[Ca^{2+}]_i$ transients ($E[Ca^{2+}]_i$) in the presence and absence of CSE inhibitors. Experimental procedures used were the same as shown in Figure 2-4A. The amplitude of $E[Ca^{2+}]_i$ in each group was determined at 5 min of reperfusion. Values are mean ± SEM; The number of myocytes sampled for calcium measurements were 9 (VP), 36 (IP), 38 (PAG+IP) and 42 (BCA+IP). ***$P < 0.001$ vs VP; $^{+++}P < 0.001$ vs IP.

2.3.4 CSE inhibitors reduced endogenous H_2S level in rat cardiomyocytes

This experiment was designed to confirm that the loss of IP cardioprotection observed in the presence of CSE inhibitors was indeed due to a decreased endogenous H_2S level in the cell. As shown in Fig. 2-6, incubating cells with PAG or BCA at 2 x 10^{-3} mol/L for 40

min, the conditions applied in the above experiments, significantly decreased H_2S production by 78.8 ± 7.1 (n = 5) and 60.4 ± 7.6% (n = 5), respectively.

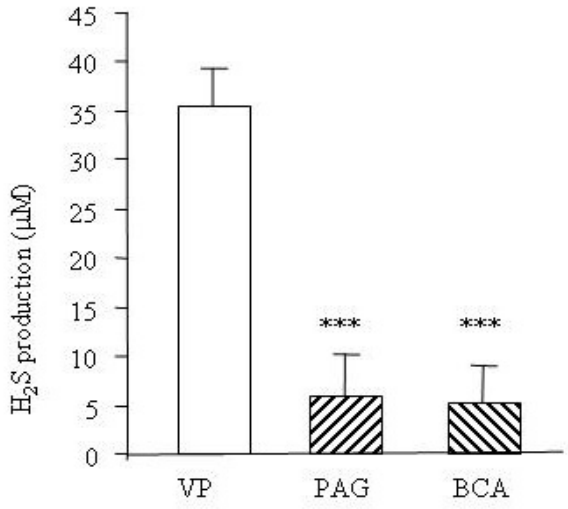

Figure 2-6 Effects of CSE inhibitors on endogenous H_2S production. Mean ± S.E.M.; n = 5 to 10. ***, $p < 0.001$ versus Con.

2.3.5 Sustained inhibition of endogenous H_2S production caused cell injury.

The above findings showed that inhibition of endogenous H_2S disabled the cardioprotection caused by IP during ischemia. However, the consequence of sustained inhibition of endogenous H_2S under physiological situation is unknown. Fig. 2-7 shows that incubation of the cardiac myocytes with PAG or BCA for 20 hours increased cellular injury index compared with the baseline level in normal cardiomyocytes. These data suggest that sustained inhibition of endogenous H_2S causes cell injury and that endogenous H_2S plays an important role in maintaining cell integrity during physiological situation.

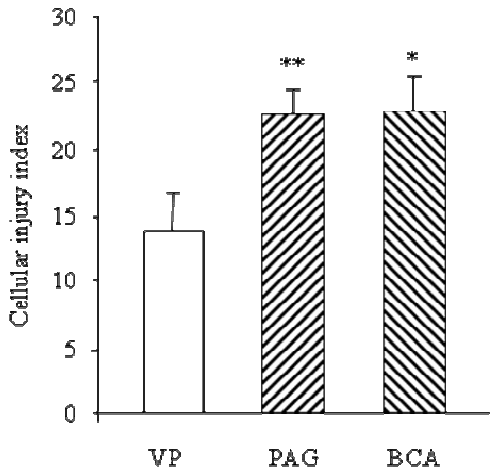

Figure 2-7 Effects of CSE inhibitors on cellular injury index. Cells were incubated with PAG and BCA for 20 hours. Mean ± SEM, N = 7-8. *P < 0.05 **P < 0.01 vs VP.

2.4 Discussion

The present study was primarily aimed to elucidate the role of endogenous H_2S in IP-induced cardioprotection. Since CSE is the main enzyme generating H_2S in the heart (Zhao et al. 2001), CSE inhibitors were used. Of the inhibitors employed, PAG has been most studied. This compound causes an irreversible, mechanism-based inhibition of CSE enzyme activity *in vitro* (Johnston et al., 1979) and, when administered to rats, produces an almost complete inhibition of liver CSE enzyme activity (measured *ex vivo*) (Porter et al., 1996; Uren et al., 1978). Similarly, BCA has also been reported to cause potent and reversible inhibition of CSE activity (Uren et al., 1978; Pfeffer et al., 1967). In the present study, we examined the effects of PAG and BCA on the endogenous H_2S production by measuring the concentration of H_2S in the culture medium. Both drugs effectively reduced the H_2S level in the culture medium, indirectly indicating a drop of intracellular H_2S level.

Based on the reliable effects of these tool drugs, the present study established an obligatory role of endogenous H_2S in the cardioprotection afforded by IP. IP protects the cardiomyocytes against ischemia-induced cell death and injury as well as impairment on cell function. Inhibition of H_2S biosynthesis with either PAG or BCA significantly diminished the protection observed in both the early and late phase of IP. Moreover, we also observed that H_2S production was suppressed in cardiomyocytes subjected to ischemia. Preconditioning the cells with brief ischemia partly restored endogenous H_2S level. Taken together, our data provide the first evidence that endogenous H_2S is necessary for the development of both early and late cardioprotection of IP.

It appears that endogenous H_2S level determines the condition of the cardiomyocytes, since decreased H_2S level is associated with ischemia and increased H_2S level by IP is associated with cardioprotection. Indeed, we found that sustained inhibition of endogenous H_2S production caused cell injury, suggesting that even under physiological situation, maintenance of certain endogenous H_2S level is important for the wellness of the cardiomyocytes.

Our results also demonstrated that IP was able to boost H_2S production. Like the role of NO in the IP cardioprotection, the increased H_2S in preconditioned cardiomyocytes is likely to contribute to the cardioprotection by activating some central components of the signaling pathway. Thus it is intriguing to observe whether direct preconditioning the cells with exogenous H_2S is sufficient to induce cardioprotection without IP.

In conclusion, the present study reveals a beneficial role of endogenous H_2S in cardiomyocytes and provokes more interest to investigate the effect of exogenous H_2S on the heart.

Chapter 3 H_2S preconditioning induces biphasic cardioprotection against ischemic injury in rat cardiomyocytes

3.1 Introduction

We investigated in this series of experiments whether pretreatment with H_2S before ischemia, namely, H_2S preconditioning (SP), is able to attenuate ischemia-associated cell damages and its underlying mechanisms.

3.2 Materials and methods

3.2.1 Experimental protocol

In the present study, H_2S preconditioning was conducted following the same protocol used for induction of IP except for the replacement of IP buffer with Kreb's buffer containing different concentrations of H_2S. For the induction of early phase of SP, cardiomyocytes were subjected to three cycle of SP and reperfusion, with each cycle composed of 3 min of SP and 5 min of reperfusion with normal Dulbecco's modified Eagle's medium (DMEM). Lethal ischemia was initiated subsequently and lasted for 9 min, followed by reperfusion for 10 min (shown in Fig 3-1A). For the induction of late phase of SP, cells were subjected to 30 min of SP. Lethal ischemia was initiated 20 hours later. To investigate the signal mechanisms for SP, cells in separated groups were treated respectively with non-selective K_{ATP} channel blocker glibenclamide (10^{-5} mol/L), sarcK_{ATP} blocker HMR-1098 (2×10^{-5} mol/L), mitoK_{ATP} bocker 5-HD (10^{-4} mol/L), NO synthase inhibitor L-NAME (10^{-4} mol/L), and PKC inhibitor chelerythrine (3×10^{-6} mol/L) 15min before and during SP. Cells in VP group were incubated with DMEM during preconditioning ischemia.

3.2.2 Other methods

Isolation of cardiomyocytes, simulation of ischemia, assessment of cell viability and morphology, intracellular Ca^{2+} imaging have been described in the *Materials and Methods* in Chapter 2.

3.2.3 Statistical analysis

Values presented are mean ± S.E.M. One-way analysis of variance was used with a post hoc (Bonferroni) test to determine the difference between groups. The significance level was set at $p < 0.05$.

3.2.4 Drugs and chemicals

L-NAME, 5-HD and chelerythrine chloride were purchased from Sigma Chemical Co, USA. HMR-1098 was a generous gift from Aventis Pharma Deutschland GmbH (Frankfurt, Germany). Glibenclamide was obtained from Tocris Cookson Ltd, UK. All chemicals were dissolved in deionized water except glibenclamide, which was dissolved in DMSO at a final concentration <0.1% (w/v).

3.3 Results

3.3.1 SP induced immediate cardioprotection in rat cardiomyocytes

Cell viability and morphology were assessed in both H_2S-preconditioned cells (SP) and vehicle-preconditioned cells (VP) 10 min into reperfusion after lethal ischemia (Fig. 3-1A). As shown in Fig. 3-1B, three cycles of 5 min of exposure to different concentrations of NaHS (10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3} mol/L) increased the percentage of nonblue cells in a concentration-dependent manner. Cell viability in SP was significantly higher than that in VP group when NaHS concentration reaches 10^{-5} mol/L, and the maximum protective response was observed at 10^{-4} mol/L of NaHS (VP, 32.6 ± 2.1%; 10^{-5} mol/L NaHS, 45.9 ± 2.3%; 10^{-4} mol/L NaHS, 47.9 ± 2.2%; all n = 7; Fig. 3-1B).

To compare the responses in term of morphology change, the percentage of rod-shaped cells was determined. As shown in Fig. 3-1C, pretreatment with NaHS at 10^{-5} mol/L and 10^{-4} mol/L preserved a greater percentage of rod-shaped cells than VP group (VP, 28.9 ± 3.3%; 10^{-5} mol/L NaHS, 41.3 ± 2.8%; 10^{-4} mol/L NaHS, 43.4 ± 3.1%; all n = 7). These data indicate that H_2S preconditioning is able to produce an IP-like effect on cell viability and morphology.

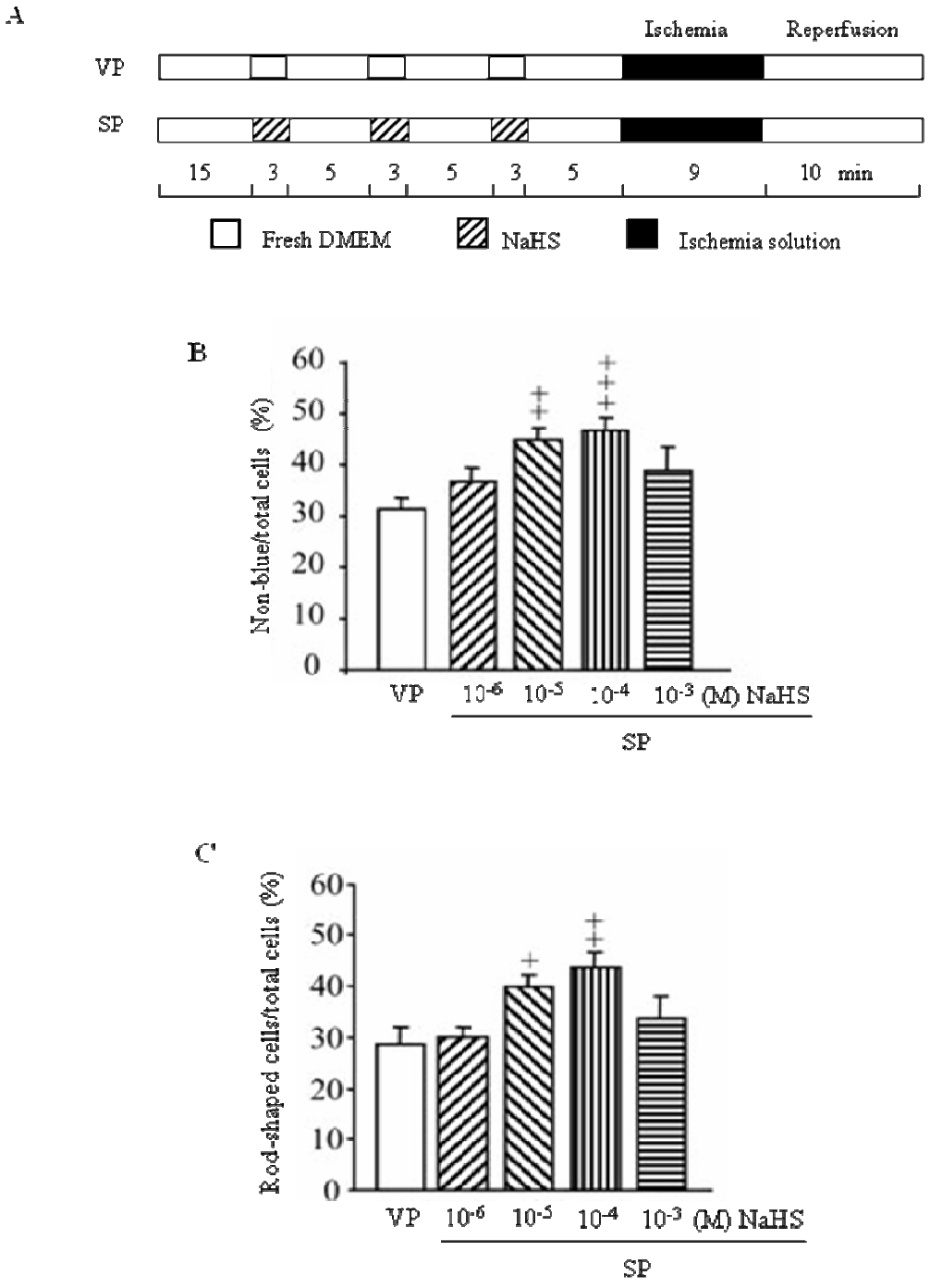

Figure 3-1 Early cardioprotection induced by SP in rat cardiomyocytes. (A) Experimental design. Solid fields, simulated ischemia; open fields, DMEM; slashed fields, DMEM containing different concentrations of NaHS. (B) Concentration-dependent effect of SP on cell viability. Nonblue cells were living cells. Mean ± S.E.M.; n = 7 cultures of 500 cells each. ++, $p < 0.01$; +++, $p < 0.001$ versus VP. (C) Concentration-dependent effect of SP on cell morphology. Rod-shaped cells per total cells were counted. Mean ± S.E.M.; n = 7 cultures of 500 cells each. +, $p < 0.05$; ++, $p < 0.01$ versus VP.

To determine the functional status of the cells, electrically induced $[Ca^{2+}]_i$ transients ($E[Ca^{2+}]_i$) before, during, and after ischemia were recorded. As shown in Fig. 3-2, the amplitude of $E[Ca^{2+}]_i$ in VP group was decreased by ischemia and reperfusion to 25.8 ± 3.0% of the amplitude of normal $E[Ca^{2+}]_i$ transients in control group. Preconditioning cells with H_2S significantly attenuated this effect, suggesting that SP improved the cell function recovery after ischemia and reperfusion injury.

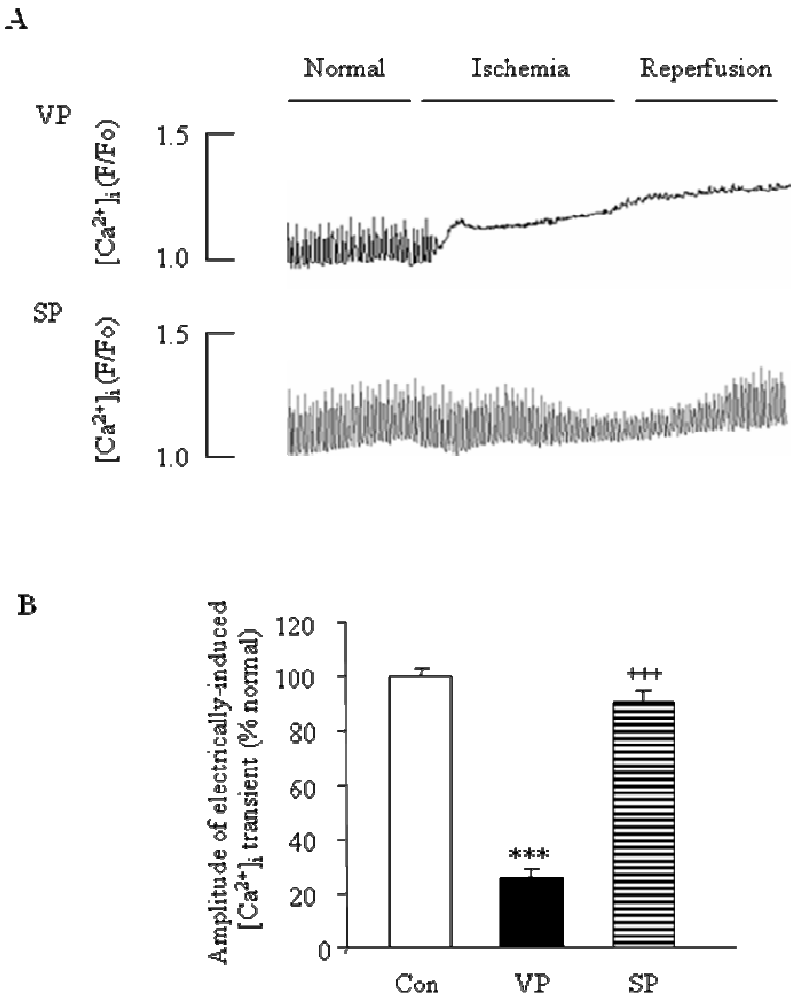

Figure 3-2 Effect of SP on recovery of electrically induced $[Ca^{2+}]_i$ transients ($E[Ca^{2+}]_i$) in single cardiomyocytes. (A) Representative tracings of $E[Ca^{2+}]_i$ in VP and SP groups. (B) Group results showing the amplitudes of $E[Ca^{2+}]_i$ in normal myocytes (Con) and myocytes in VP and SP groups at 10 min into reperfusion. Values are mean ± S.E.M.; $n = 25$. ***, $p < 0.001$ versus the value in the Con group; +++, $p < 0.001$ versus the value in the VP group.

3.3.2 SP induced late cardioprotection in rat cardiomyocytes

To investigate whether H_2S was able to produce late cardioprotection against ischemic injury, cardiomyocytes were subjected to lethal ischemia 20 hours after they were preconditioned with different concentrations of H_2S (Fig. 3-3A). After 5 min of severe ischemia and 10 min of reperfusion, the percentage of non-blue cells was 21.4 ± 7.0% (N = 15) in VP group (Fig. 3-3B). In SP groups where cells were pretreated with 10^{-6}~10^{-3} mol/L NaHS for 30 min, the percentage of non-blue cells was significantly higher than that of VP group when the NaHS concentration was 10^{-5} mol/L and the maximum protective response was observed at a concentration of 10^{-4} mol/L (38.5 ± 4.9%, N = 9).

A similar result was found when using the percentage of rod-shaped cells as an indicator of the cell conditions. Only 10.8 ± 1.7% (N = 10) of the cells were rod-shaped in VP group. NaHS at 10^{-5} ~ 10^{-4} mol/L significantly increased the percentage of rod-shaped cells. The maximum protection reached at 10^{-4} mol/L NaHS (16.4 ± 2.0%, N = 11; Fig. 3-3C). These dose-dependent responses are consistent with those found in the early phase of SP-induced cardioprotection, indicating that SP is able to induce biphasic cardioprotection against ischemic injury.

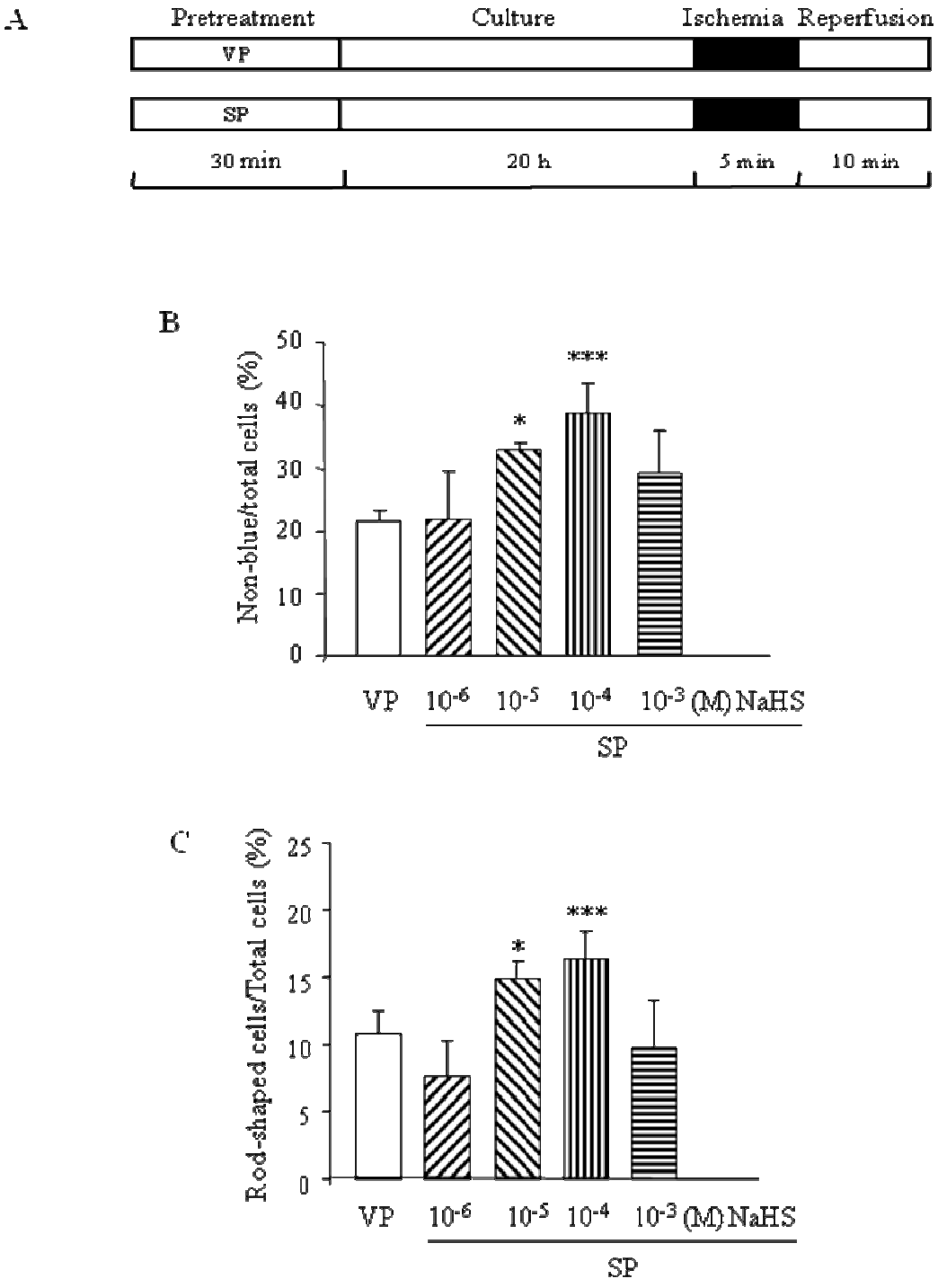

Figure 3-3 Late cardioprotection induced by SP in rat cardiomyocytes. (A) Experimental protocol. Ischemia was induced 20 hours after preconditioning. (B) Concentration-dependent effect of NaHS on cell viability. Values are presented as non-blue cells per total myocytes counted. (C) Concentration-dependent effect of NaHS on cell morphology. Values are presented as rod-shaped cells per total myocytes counted. All data are mean ± SEM; N = 5-15 cultures of 200-500 cells each. *P < 0.05, ***P < 0.001 vs VP.

3.3.3 SP-induced late cardioprotection lasted at least 28h

Because of the clinical significance of the late cardioprotection, we investigated the time course of the protective effects by determining cell viability and morphology at different time points (1, 6, 16, 20, and 28 hours) after 30 min of exposure to H_2S (Fig. 3-4A). Despite decreased viability with increased culture period, SP groups at 1, 16, 20 and 28 hours still displayed higher percentage of nonblue cells compared with time-matched VP groups (Fig. 3-4B). Similar results were also found when examining cell morphology (Fig. 3-4C). These data confirmed that there are two windows of cardioprotection induced by H_2S preconditioning and also further demonstrated that SP-induced late cardioprotection appeared 16 hours after preconditioning and lasts at least until 28 hours.

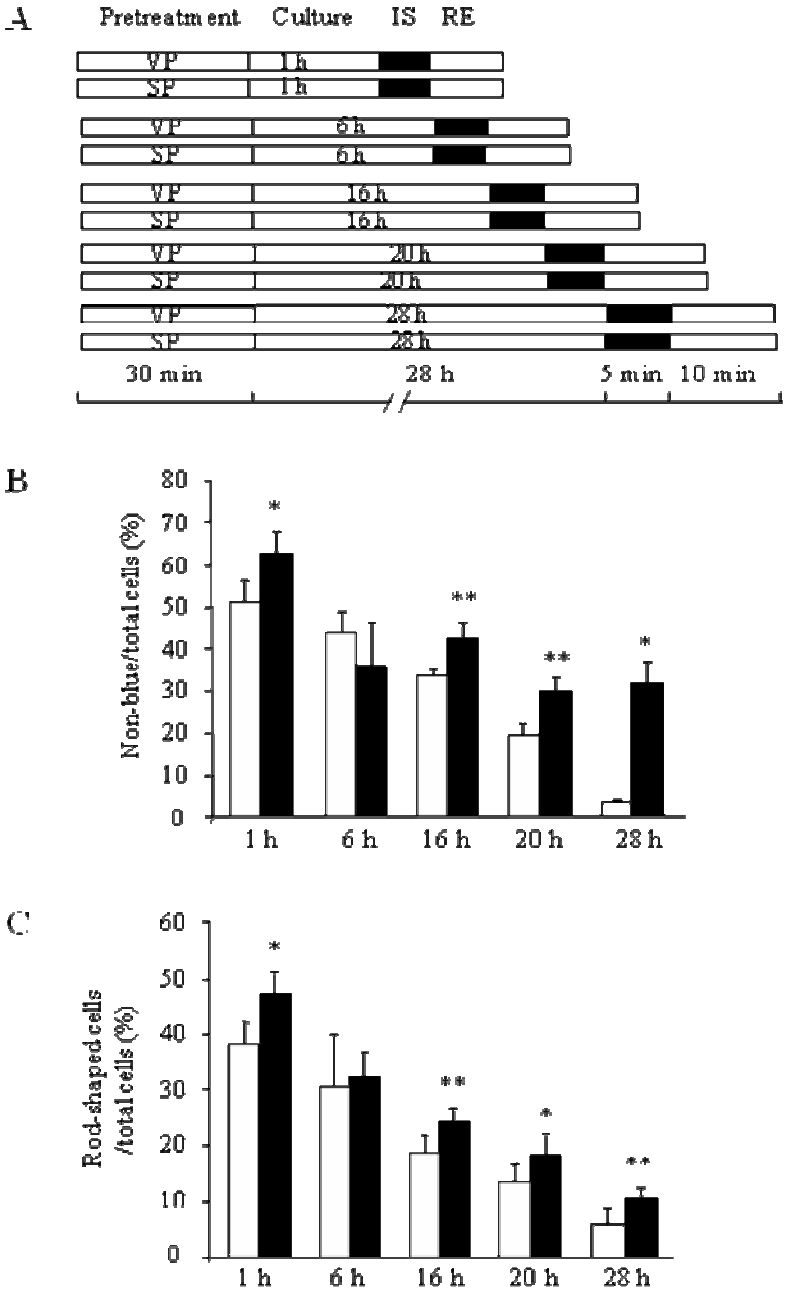

Figure 3-4 Time course of SP-induced cardioprotection. (A) Experimental protocol. After pretreatment, cells were cultured in DMEM for 1, 6, 16, 20, and 28 hours respectively before being subjected to severe ischemia (IS) for 5 min followed by 10min of reperfusion (RE). Cell viability (B) and cell morphology (C) were examined at 10 min into reperfusion. Values are presented as mean ± SEM; N = 5-16 cultures of 200~600 cells each. *P < 0.05, **P < 0.01vs corresponding VP groups.

3.3.4 SP-induced late cardioprotection counteracts different periods of ischemia and reperfusion

These series of experiments were designed to determine the effectiveness of SP-induced late cardioprotection against different periods of ischemia and reperfusion. As shown in Fig. 3-5B&C, after 5 min, 10 min or 30 min of ischemia and 10 min of reperfusion, cell viability and percentage of rod-shaped cells in SP groups were significantly higher than those in corresponding VP groups.

We also observed the cardioprotective effects of SP at different time points during reperfusion. SP increased myocyte viability and percentage of rod-shaped cells at 10 min, 20 min or 60 min into reperfusion (Fig. 3-6). These data suggest that SP protects cardiomyocytes from different periods of ischemic insults and the cardioprotection lasts at least 60 min after the onset of reperfusion.

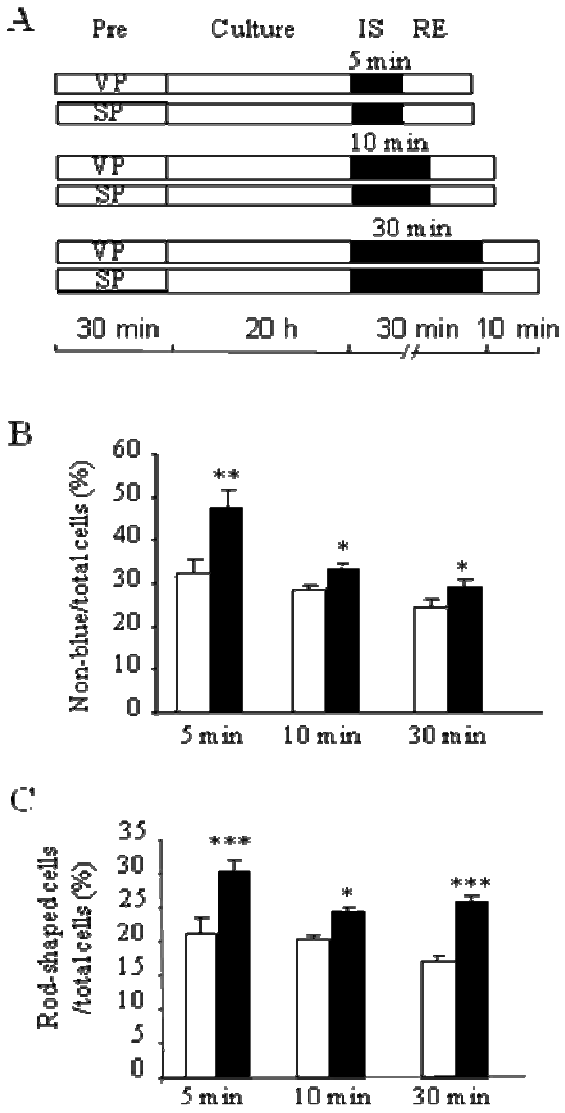

Figure 3-5 Late cardioprotection induced by SP against different periods of ischemia. (A) Experimental protocol. Cells were subjected to different periods of ischemia (IS) after 20 hours culture. Cell viability (B) and cell morphology (C) were examined at 10 min into reperfusion (RE). Values are presented as mean ± SEM; N = 5-16 cultures of 200~600 cells each. *P < 0.05, **P < 0.01, ***P < 0.001 vs corresponding VP groups.

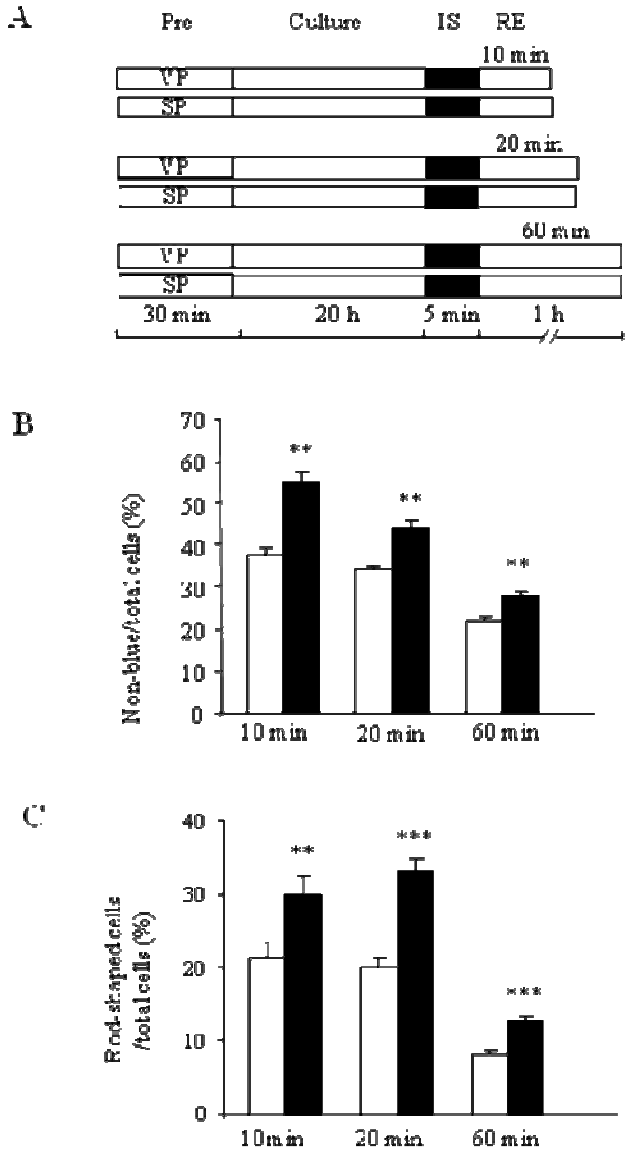

Figure 3-6 Late cardioprotection induced by SP against different periods of reperfusion. (A) Experimental protocol. After 20 hours of culture, cardiomyocytes were subjected to 5 min of ischemia (IS) followed by different periods of reperfusion (RE). Cell viability (B) and cell morphology (C) were examined at 10 min, 20 min or 1 hour into reperfusion (RE). Values are presented as mean ± SEM; N = 5-16 cultures of 200~600 cells each. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs corresponding VP groups.

3.3.5 SP-induced late cardioprotection was blocked by K_{ATP} inhibitors

The goal of this series of experiments was to probe the mechanism(s) involved in the late cardioprotection of SP. The experimental procedures were shown in Fig. 3-7A. Treatment with glibenclamide (10^{-5} mol/L), a non-selective K_{ATP} blocker, 15 min before and during SP significantly attenuated the cardioprotection observed in SP groups, as manifested by the reduced cell viability and increased cellular injury (LDH release) compared with SP groups (Fig. 3-7B&C). The contribution of each subtype of K_{ATP} channels was also assessed by using subtype-specific blockers, 5-HD (10^{-4} mol/L, a mitochondrial K_{ATP} blocker), and HMR-1098 (2×10^{-5} mol/L, a sarcolemmal K_{ATP} blocker). As shown in Fig. 3-7B&C, HMR-1098 but not 5-HD reversed SP-induced protective effects on cell viability and injury, suggesting that only sarcolemmal K_{ATP} plays a part in the cardioprotection of SP. In separate experiments, glibenclamide, 5-HD or HMR-1098 alone (at the same concentration and over the same time course) did not affect cell viability.

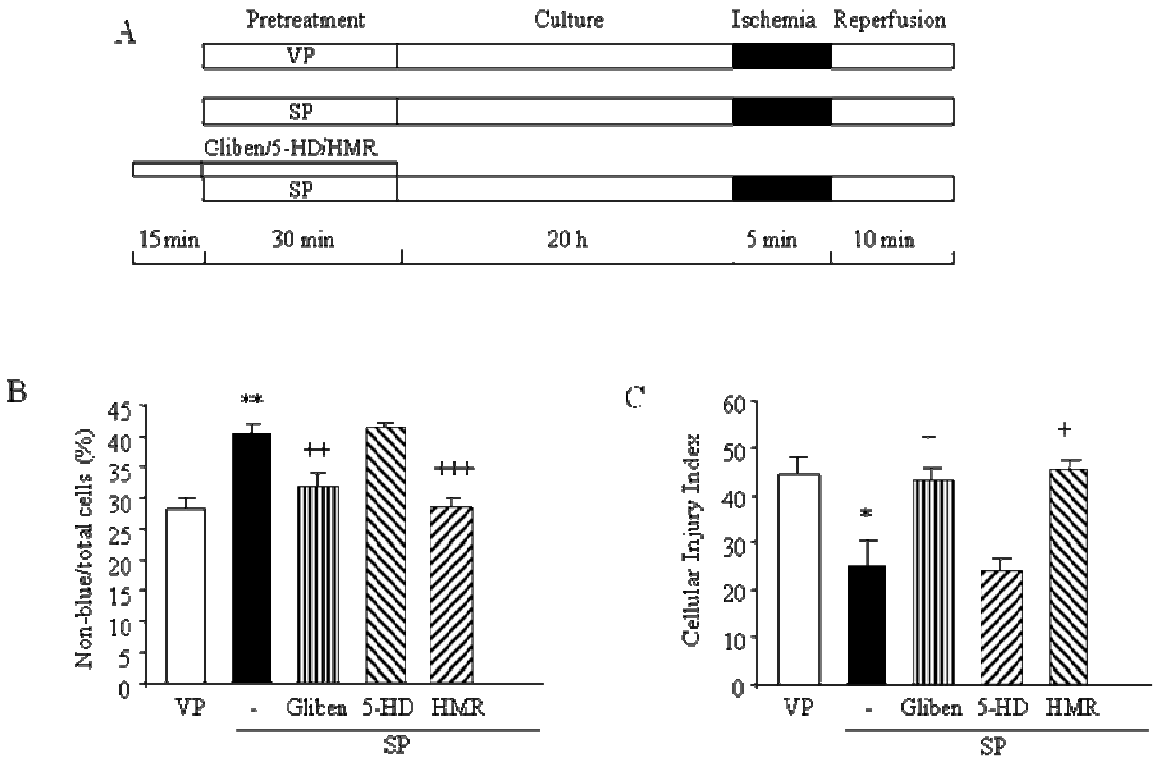

Figure 3-7 Effects of SP on cell viability and cellular injury in the presence and absence of K_{ATP} channel blockers. (A) Experimental design. Glibenclamide (Gliben), 5-HD and HMR-1098 (HMR) were applied 15 min before and during SP. (B) Cell viability. Values were presented as non-blue cells per total myocytes counted; n=6-7 cultures of ≈200-500 cells each. (C) Cellular injury index. Values were presented as supernatant LDH activity/total LDH activities (supernatant + cells lysate). $N = 5$. Mean ± SEM. * $P < 0.05$, ** $P < 0.01$ vs VP; $^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.001$, vs SP.

To determine whether K_{ATP} channels are also involved in the protection of SP on cell function, we observed the electrically-induced $[Ca^{2+}]_i$ transients during ischemia and reperfusion in myocytes co-pretreated with H_2S and K_{ATP} blockers. Fig. 3-8 shows that the protective effect of SP was significantly attenuated by glibenclamide and HMR-1098, while 5-HD did not produce any significant effect.

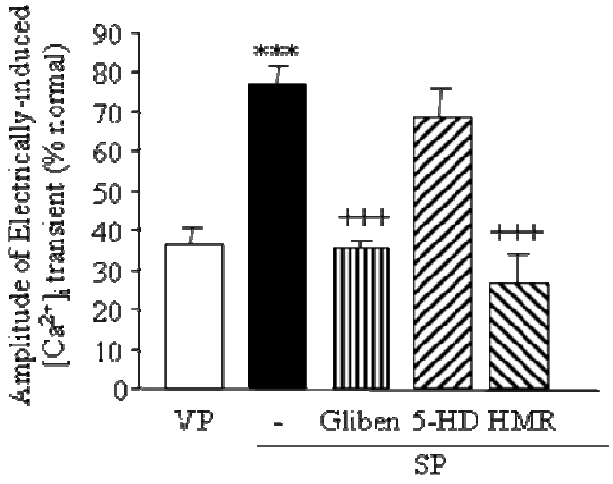

Figure 3-8 Effects of SP on recovery of $E[Ca^{2+}]_i$ amplitude in the presence and absence of K_{ATP} channel blockers. The amplitude of $E[Ca^{2+}]_i$ was determined at 10 min into reperfusion. Values are mean ± SEM; The numbers of myocytes sampled for measurements were 22 (VP), 20 (SP), 38 (Gliben + SP), 16 (5-HD + SP) and 12(HMR + SP). ***$P < 0.001$ vs VP; $^{+++}P < 0.001$ vs SP.

3.3.6 SP-induced late cardioprotection was blocked by a NO synthase inhibitor

To examine the involvement of NO in the cardioprotection of SP, L-NAME (10^{-4} mol/L) was used as a non-selective inhibitor of all NO synthase. L-NAME significantly attenuated the cardioprotection of SP on cell viability (shown in Fig. 3-9A) and cell function (shown in Fig.3-9B).

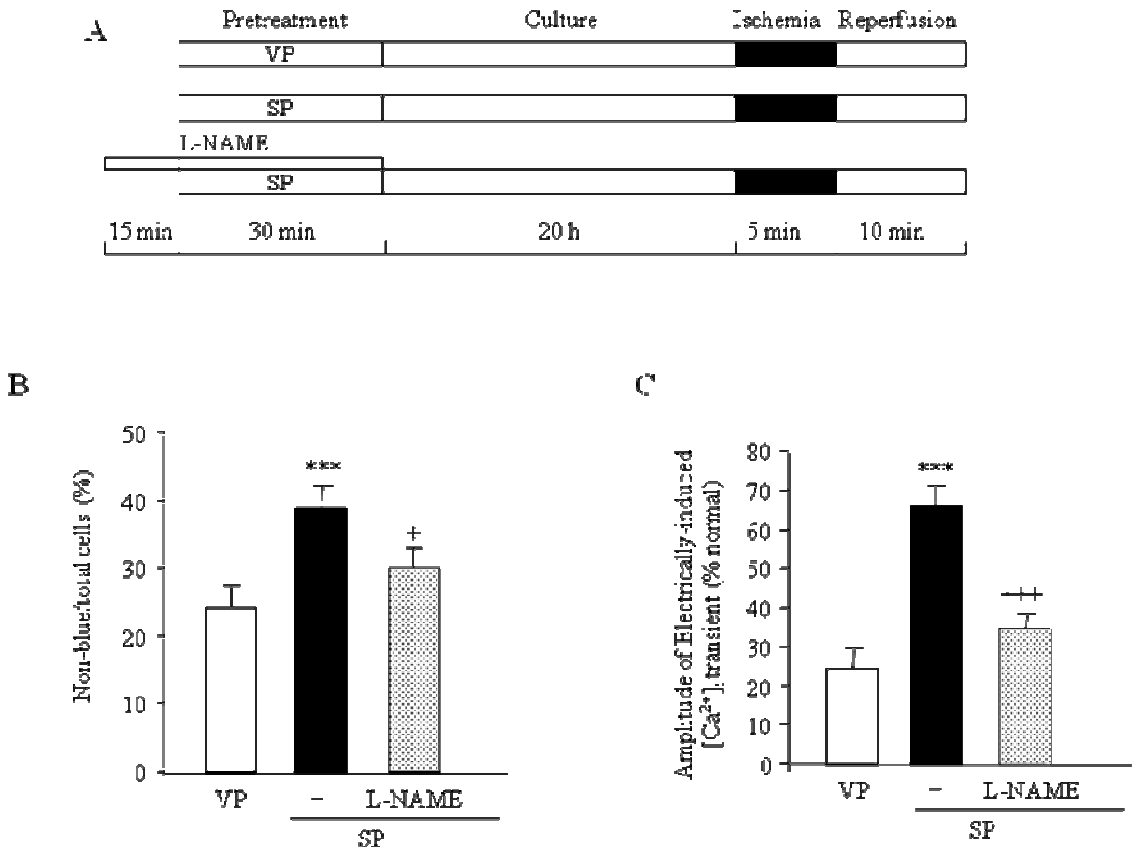

Figure 3-9 Effects of SP on cell viability and E$[Ca^{2+}]_i$ transients in the presence and absence of a NO synthase inhibitor, L-NAME. (A) Experiment protocol. (B) Cell viability at 10 min into reperfusion. Mean ± SEM; N = 6-7 cultures of ≈200-500 cells each. (C) Amplitude of E$[Ca^{2+}]_i$ transients at 10 min into reperfusion. The number of myocytes sampled for calcium measurements were 22 (VP), 39 (SP) and 46 (L-NAME+SP). ***$P < 0.001$ vs VP; $^{+}P < 0.05$, $^{+++}P < 0.001$ vs SP.

3.3.7 SP-induced late cardioprotection was blocked by PKC inhibitors

To determine the role of PKC in the SP-induced cardioprotection, PKC inhibitors, chelerythrine (3 x 10^{-6} mol/L, chelery) and calphostine C (10^{-7} mol/L, Cal C) were applied 15 min before and during SP preconditioning (Fig. 3-10A). Both drugs, which alone had no effects, blunted the cardioprotection of SP on cell viability and cellular injury (Fig.3-10B&C).

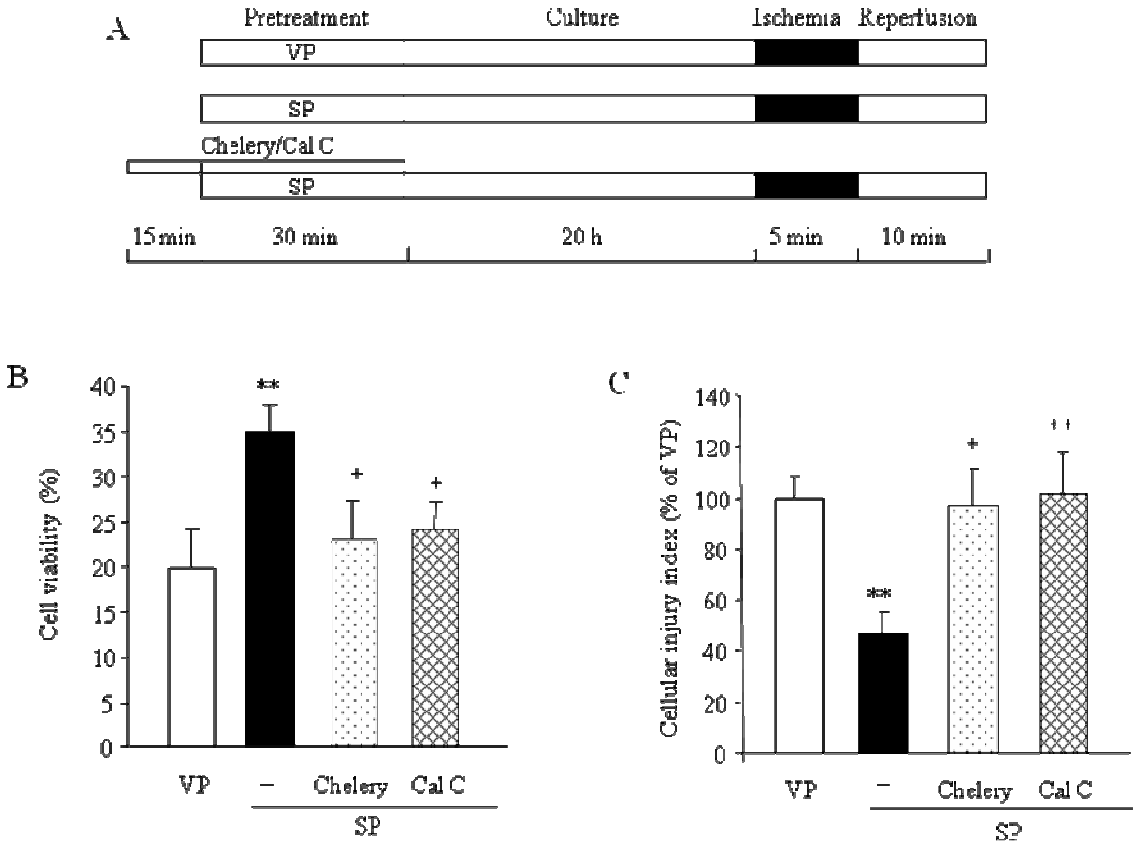

Figure 3-10 Effects of SP on cell viability and cellular injury in the presence and absence of PKC inhibitors. (A) Experiment protocol. Chelerythrine (Chelery) and Calphostine C (Cal C) were apllied 15 min before and during SP. (B) Cell viability. Values were presented as non-blue cells per total myocytes counted. n=5-13 cultures of ≈200-500 cells each. $^{**}P<0.01$ vs VP; $^{+}P<0.05$ vs SP. (B) Cellular injury index. Values were presented as supernatant LDH activity/total LDH activities (supernatant + cell lysate) normalized to 100% of VP group (control). n=6. Mean ± SEM; $^{**}P<0.01$ vs VP, $^{+}P<0.05$, $^{++}P<0.01$ vs SP.

3.4 Discussion

In the present study, we investigated the effect of exogenous H_2S on rat cardiomyocytes undergoing lethal ischemia. We found that NaHS, a donor of H_2S, produced biphasic cardioprotection against ischemia-caused damages. The first phase of cardioprotection occurred immediately after H_2S preconditioning, while the second phase is observable between 16 hours and 28 hours after H_2S preconditioning. Due to the limited time that cardiomyocytes can survive in vitro, it is technically difficult to observe the late phase of cardioprotection after 28 hours. However, it is very likely that the late cardioprotection could last much longer, as the protective effect was still very strong at 28 hours. These data suggest that SP follows a similar time course as IP, which also produces immediate (1 to 2 hours) and delayed (12 to 72 hours) protection against ischemia (Murry et al., 1986; Kuzuya et al., 1993; Marber et al., 1993).

In both phases, NaHS at 10^{-5}-10^{-4} mol/L concentration-dependently increased the cell viability and the percentage of rod-shaped cells. The maximum protective effect was always observed at 10^{-4} mol/L NaHS. Accordingly, we used the concentration 10^{-4} mol/L for all subsequent *in vitro* experiments.

Considering the greater clinical importance of the late cardioprotection, we performed detailed study on its effectiveness against I/R injury in varied experimental settings. We found that H_2S preconditioning protected cardiomyocytes against different periods of ischemia. As the duration of ischemia increases, the protection appears more significant on cell morphology than on cell viability, which implies that SP-induced cardioprotection is particularly effective in salvaging more percentage of functional cells rather than living cells. Similar results were also found when cell viability and cell morphology were

determined after different periods of reperfusion. The pronounced protection observed in SP group after 60 min of reperfusion indicates that the effect of H_2S preconditioning is prevention instead of delay of cell death.

In an attempt to probe the signaling mechanism underlying the late cardioprotection, we determined the involvement of K_{ATP} channels, NO and PKC due to their important roles in the late cardioprotection of IP. There are two separate populations of K_{ATP} channels within the myocardium: the sarcK_{ATP} and mitoK_{ATP} channels (Gross and Fryer, 1999). Both sarcK_{ATP} channels and mitoK_{ATP} channels have been reported to trigger or mediate the cardioprotective effects of IP (Gross and Peart, 2003). Initial evidence suggested that the sarcK_{ATP} channels triggered or mediated the cardioprotective effects of IP (Jovanovic et al., 1986; Toyoda et al., 2000); however, more recent findings have suggested a major role for mitoK_{ATP} channels (Gross and Peart, 2003; Liu et al., 1998; Liu et al., 1999). In this study, we found that both non-selective K_{ATP} channel blocker glibenclamide and selective sarcK_{ATP} blocker HMR-1098 reversed the cardioprotection of SP, while selective mitoK_{ATP} blocker 5-HD failed to affect the protection of SP. These data clearly indicate that sarcK_{ATP} is the subtype of K_{ATP} channel that mediates the SP-induced late cardioprotection. Opening of sarcK_{ATP} channels is associated with potassium efflux, depolarization of cell membrane and shortening of APD (Noma, 1983; Cole et al., 1981). These effects reduce Ca^{2+} influx via L-type Ca^{2+} channels and prevent the reversal of the Na^{+}-Ca^{2+} exchange system. The resultant decrease in Ca^{2+} influx would be expected to lead to a reduction of the mechanical contraction, blunting of intracellular Ca^{2+} overload, and energy sparing during ischemia and early reperfusion.

The present study also demonstrated an essential role of PKC in SP-induced late cardioprotection with two different PKC inhibitors. The involvement of PKC is probably secondary to the opening of K_{ATP} channels, since H_2S has been shown to directly open K_{ATP} channels in smooth muscle cells (Zhao et al., 2001). However, activation of PKC and K_{ATP} channel could also be codependent (Baxter et al., 1995; Gross and Peart, 2003), given that protection provided by direct K_{ATP} channel openers could be abolished by PKC antagonists and *vice versa* (Gaudette et al., 2000). Additional experiments are needed to determine whether opening of K_{ATP} channels is an event upstream of PKC activation in SP-induced late cardioprotection. In addition, the two PKC inhibitors used, chelerythrine and calphostin C, are not isoform-selective, which warrants further studies to determine the specific isoforms involved.

The role of NO in the SP-induced cardioprotection is another focus of interest in this study. Over the past decade, many studies have revealed a critical role of NO in IP-induced cardioprotection (Bolli, 2001). Importantly, NO alone is also sufficient to induce late cardioprotection against myocardial ischemia (Takano et al., 1998). In the present study, we found that inhibition of NO synthesis with L-NAME significantly attenuated the cardioprotection of H_2S on both cell viability and cell function. This is another line of evidence supporting the concept that crosstalk exits between endogenous gasotransimitters (Szabó, 2007).

In conclusion, the present study has demonstrated, for the first time, that pharmacological preconditioning with the H_2S donor NaHS is able to confer cardioprotection against ischemia probably via activation of sarcK_{ATP} channel, PKC and release of NO.

Chapter 4 H_2S preconditioning-induced PKC activation regulates intracellular calcium handling in rat cardiomyocytes

4.1 Introduction

The study in Chapter 3 has revealed that PKC is involved in the SP-induced cardioprotection. The PKC family consists of at least 10 isoforms, of which PKC-α, ε, and δ are the prominent isoforms expressed in the heart (Mackay and Mochly-Rosen, 2001). Upon stimuli, PKC isoforms translocate from the cytosol to subcellular membrane regions, a process associated with their activation (Mackay and Mochly-Rosen, 2001). However, it is completely unknown which isoforms could be activated by H2S preconditioning and how they mediate or execute the cardioprotection.

PKC activation has been reported to play a role in regulating intracellular calcium handling (Ladilov et al., 1998; Stamm et al., 2001; Stamm and del Nido, 2004). Under the physiological condition, intracellular calcium concentration is sophisticatedly regulated by several proteins present in the sarcolemmal and sarcoplasmic reticulum (SR) membranes. Upon the arrival of action potential, Ca^{2+} influxes through the L-type Ca^{2+} channel and triggers the opening of the ryanodine receptor (RyR), resulting in further release of Ca^{2+} from SR, which accomplishes the sharp $[Ca^{2+}]_i$ elevation required for myofibril contraction (Guatimosim et al., 2002). In the rat cardiomyocytes, >90% of the Ca^{2+} after contraction is immediately uptaken by SR via sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA), while the remaining Ca^{2+} is pumped out of the cell via Na^{+}/Ca^{2+} exchanger (NCX) (Bers, 2000).

However, the well-controlled intracellular Ca^{2+} homeostasis could be easily disrupted by ischemia and reperfusion insults. During ischemia, excessive Ca^{2+} accumulates in the cytosol (Piper et al., 1993) and leads to a series of severe damages upon reperfusion. For example, once re-energized by reperfusion, cardiac myofilaments contract in an extreme and sustained manner (hypercontracture) due to overstimulation of calcium on the contractile apparatus (Siegmund, 1993). In single cardiomyocytes, such hypercontracture causes irreversible shortening of cell length. In tissues, it causes a disruptive change in myocardium termed contraction band necrosis (Ganote, 1983). Under this circumstance, a faster clearing of excessive Ca^{2+} from cytosol is therapeutically important as it would potentially attenuate Ca^{2+} overloading during ischemia (Abdallah et al., 2005) and prevent subsequent damages. Since PKC is implicated in the intracellular Ca^{2+} handling, it is worthwhile investigating whether H_2S could alter intracellular Ca^{2+} handling via activation of PKC.

Taken together, there is no information so far available regarding the isoforms of PKC involved in H_2S preconditioning and how the cardioprotective signals are conveyed forwards. Thus in the current chapter, a close inspection on PKC was performed with an emphasis on its upstream and downstream connections in the signal transduction pathway of H_2S preconditioning.

4.2 Materials and Methods

4.2.1Experimental protocol

Myocytes were subjected to H_2S preconditioning (SP) by incubation with 10^{-4} mol/L NaHS for 30 min. 20 hours later, samples were harvested for western blotting experiment or for intracellular Ca^{2+} transient recording. To study the sequence of

signaling events between PKC activation and K_{ATP} opening, cells were treated with glibenclamide (10^{-5} mol/L, a blocker of K_{ATP} channel) 15 min before and during SP. In separate experiments, preconditioned cells were subjected to severe ischemia followed by 10min's reperfusion with normal medium. Resting Ca^{2+} was traced real-time during ischemia for examination on cytosolic Ca^{2+} accumulation. Cell lengths before ischemia and after the onset of reperfusion were compared for evaluation of hypercontracture.

4.2.2 Cell fractionation and western blotting

A cell fractionation technique was adopted from the literature (Mackay and Mochly-Rosen, 2001; Weber et al., 2005). After 20h's incubation, cardiomyocytes were lysed with 150 μl ice-cold lysis buffer containing 125mM NaCl, 25mM Tris pH7.5, 5mM EDTA, 1% NP-40 and protease inhibitors and shaken on ice for 1h. The cell lysate was centrifuged at 1000×g at 4^{o}C for 10 min for rough partition between cytosolic and membrane fractions. The supernatant was recentrifuged at 16000×g at 4^{o}C for 15min to get rid of contaminating pellet materials and collected as cytosolic fraction. The initial pellets were resuspended in 100 μl cell lysis buffer containing 1% triton X-100 and shaken on ice for another 60min and were then centrifuged at 16000×g at 4^{o}c for 15min. The second supernatant was collected as membrane fraction. Epitopes were exposed by boiling the protein samples at 90^{o}c water for 5 min. Each fraction was analyzed for protein content by Bradford assay. Equal amounts of protein were loaded and electrophoresed with 10% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Amersham Biosciences, U.K.). The membrane was probed with antibody against PKCε (Santa Cruz Biotechnology, Inc., California, U.S.A.), PKCα and

PKCδ (Cell Signaling Technology, Inc., Danvers, U.S.A). Immunoreactivity was detected using an ECL advance western blot detection kit (Amersham Biosciences, U.K.).

4.2.3 Measurement of $[Ca^{2+}]_i$

Electrically induced $[Ca^{2+}]_i$ transients ($E[Ca^{2+}]_i$) were generated by stimulating myocytes with a stimulator at 0.2 Hz (Grass S88). Caffeine-induced $[Ca^{2+}]_i$ transients ($C[Ca^{2+}]_i$) were generated by adding 10^{-2} mol/L caffeine directly to the incubation buffer. Resting Ca^{2+} level was recorded during ischemia challenge without any electrical or caffeine stimulations.

4.2.4 Measurement of cell length

Cardiomyocytes were placed on the stage of an inverted microscope (Nikon TE2000-S). The cell image was taken with a digital camera (Nikon DS-5M-L1) connected to the microscope with a 20X objective and analyzed with NIS-documentation software (Nikon).

4.2.5 Statistical analysis

Values presented are mean ± SEM. Statistic comparisons were performed by one-way ANOVA and bonferroni for post-hoc analysis. The significance level was set at $P<0.05$.

4.3 Results

4.3.1 SP promoted translocation of PKC α,ε and δ to membrane fraction

To determine the activated PKC isoforms in the delayed phase of cardioprotection induced by SP and IP, subcellular distributions of three main PKC isoforms present in the heart, α, ε and δ, were examined with western blotting experiments 20h after SP and IP. As shown in Fig. 4-1, SP induced all three isoforms of PKC translocation from cytosol to

membrane. The membrane/cytosol ratios of PKC-α,ε and δ abundance increased approximately two folds in SP group compared with VP group (Fig. 4-1D-F). Interestingly, IP only induced translocation of PKCε and δ, but had no effect on PKCα translocation. These data suggest that the SP and IP may employ different subsets of PKC isoforms to mediate their cardioprotection.

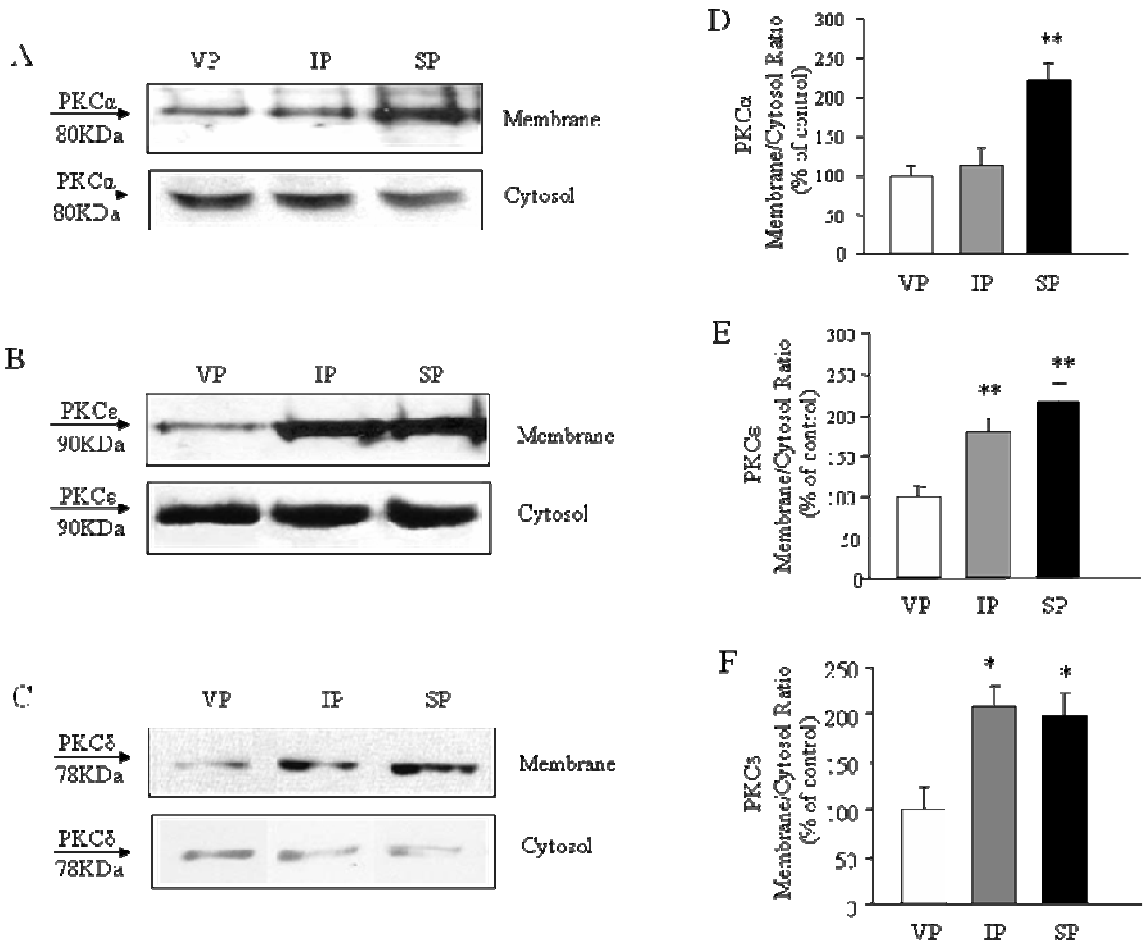

Figure 4-1. Effects of IP and SP on subcellular distribution of PKCα (A&D), PKCε (B&E), and PKCδ (C&F). (A-C) Representatives of five separate experiments for each isoform. (D-F) Gourp results of membrane/cytosol ratio of PKC isoform abundance. They are calculated by relative densitometry and normalized to 100% of VP group. The data are presented as mean ± SEM, n=5, **P*<0.05 ***P*<0.01 vs VP.

4.3.2 K_{ATP} channel blocker prevented translocation of PKCε

Glibenclamide (10^{-5} mol/L), a K_{ATP} channel blocker, was used to examine whether SP-induced translocation of PKC isoforms was secondary to the opening of K_{ATP} channels. As shown in Fig. 4-2, glibenclamide blocked SP-induced translocation of PKCε but did not affect translocation of PKCα and PKCδ, suggesting that only PKCε among the three isoforms is downstream to K_{ATP} channels in the signaling pathway of SP.

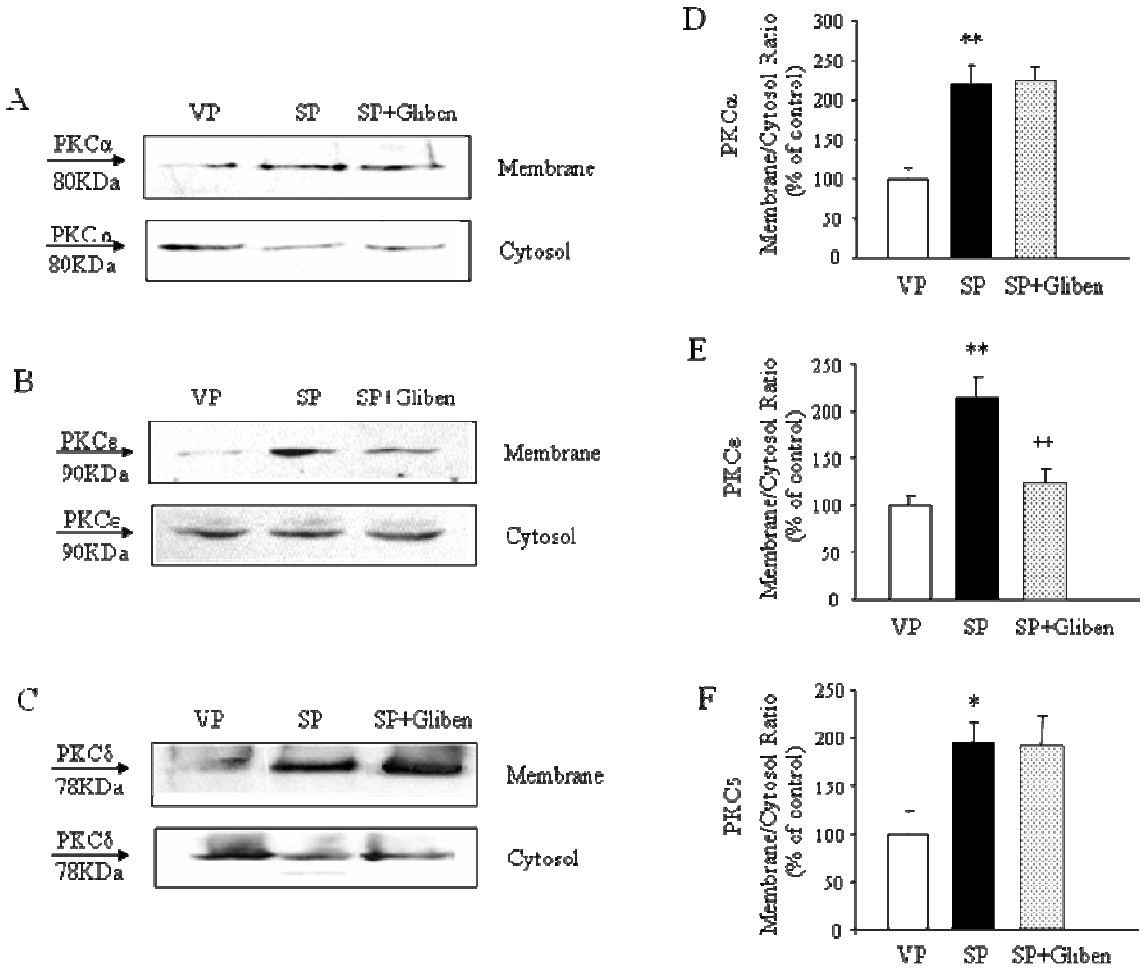

Figure 4-2. Effects of K_{ATP} blockers on SP-induced translocation of PKC isoforms. Glibenclamide was applied 10 min before and during SP treatment. (A-C) Representatives of five separate experiments for each isoform. (D-F) Correspondent membrane/cytosol ratios calculated by relative densitometry and normalized to 100% of VP group. The data are presented as mean ± SEM, n=5~7, $^{*}P<0.05$ $^{**}P<0.01$ vs VP, $^{++}P<0.01$ vs SP.

4.3.3 SP accelerated SR-Ca^{2+} uptake rate in a PKC-dependent manner

The decline rate of $[Ca^{2+}]_i$ is mainly determined by Ca^{2+} uptake to SR via SERCA, which is responsible for the removal of ~90% Ca^{2+} from the cytosol (Bers, 2000). Thus t_{50} (half-decay time) and t_{90} (90% decay time) of $E[Ca^{2+}]_i$ were measured as indicators of SR uptake rate. As shown in Figure 4-3, both t_{50} and t_{90} were significantly shortened in SP group, compared with those observed in VP. Co-treatment with 3 x 10^{-6} mol/L chelerythrine during SP reversed this effect, suggesting that SP accelerated the rate of SR-Ca^{2+} uptake through a PKC-denpendent pathway.

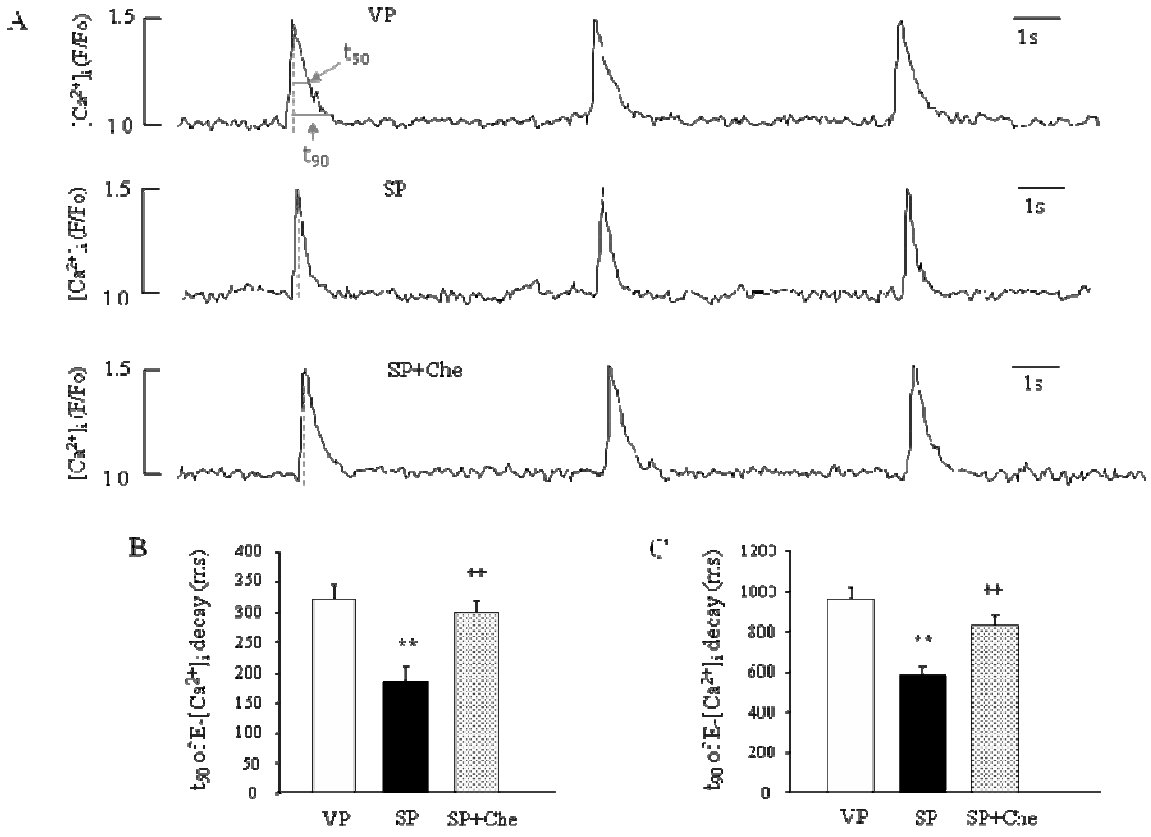

Figure 4-3. Effects of SP on SR-Ca^{2+} uptake rate in single ventricular myocytes in the presence and absence of PKC inhibitor. (A) Typical transients of $E[Ca^{2+}]_i$ in VP, SP and SP+Chelerythrine (SP+Che). Half-decay time t_{50} (B) and 90% decay time t_{90} (C) of $E[Ca^{2+}]_i$ indicate the rate of Ca^{2+} uptake to SR via SERCA. The data are presented as mean ± SEM, n=14(VP), 11(SP), 7(Che). **P*<0.05 vs VP, ***P*<0.01 vs VP, $^{+}$*P*<0.05 vs SP. $^{++}$*P*<0.01 vs SP.

4.3.4 SP accelerated Ca^{2+} extrusion rate in a PKC-dependent manner

Because caffeine keeps RyR open, SR is unable to sequester Ca^{2+} during its application. The decline of $[Ca^{2+}]_i$ therefore depends on Ca^{2+} extrusion through NCX (Sham et al., 1995). The rate of extrusion can be reflected by decay of $C[Ca^{2+}]_i$ (t_{50} and t_{90}). As shown in Figure 4-4, SP significantly shortened both t_{50} and t_{90} of the decay of $C[Ca^{2+}]_i$. These effects were reversed by inhibition of PKC with chelerythrine (3×10^{-6} mol/L). We also examined the SR-Ca^{2+} load by measuring the amplitude of $C[Ca^{2+}]_i$ since caffeine depletes the intracellular Ca^{2+} store at a burst. The amplitude did not differ between groups, which excluded a less SR-Ca^{2+} load as a cause of the faster clearing of cytosolic Ca^{2+}.

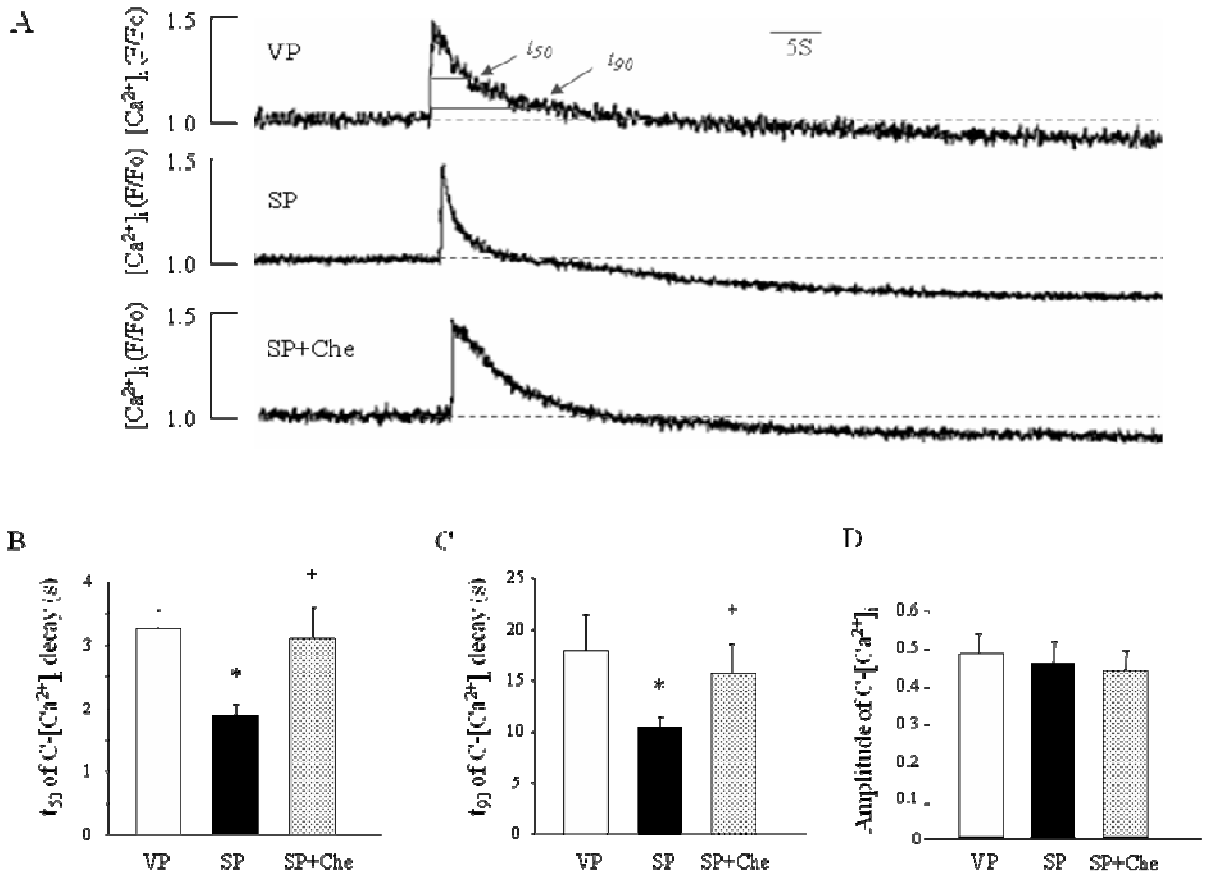

Figure 4-4. Effects of SP on Ca^{2+} extrusion rate in single ventricular myocytes in the presence and absence of PKC inhibitor. (A) Typical transients of $C[Ca^{2+}]_i$ in VP, SP and SP+Chelerythrine (SP+Che) Half-decay time t_{50} (B) and 90% decay time t_{90} (C) of $C[Ca^{2+}]_i$ indicate the rate of Ca^{2+} extrusion via NCX. Amplitude (D) of $C[Ca^{2+}]_i$ reflects the Ca^{2+} load in SR. The data are presented as mean ± SEM, n=13(VP), 9(IP), 13(SP), 8(Che), 12(Gliben). **P*<0.05 vs VP, ***P*<0.01 vs VP, ***P*<0.001 vs VP, $^{++}$*P*<0.01 vs SP.

4.3.5 SP attenuated cytosolic Ca^{2+} accumulation during ischemia in a PKC-dependent manner

Resting Ca^{2+} elevation was traced during ischemia to investigate whether the Ca^{2+} handling altered by SP affected the Ca^{2+} accumulation in the cytoplasm during ischemia challenge. As shown in Fig. 4-5, the resting Ca^{2+} level in VP increased dramatically within 15 min of ischemia, indicating a severe Ca^{2+} accumulation in the cytosol.

However, the increase of resting Ca^{2+} in SP was limited to a much lower extent. Pretreatment with chelerythrine during SP diminished this effect.

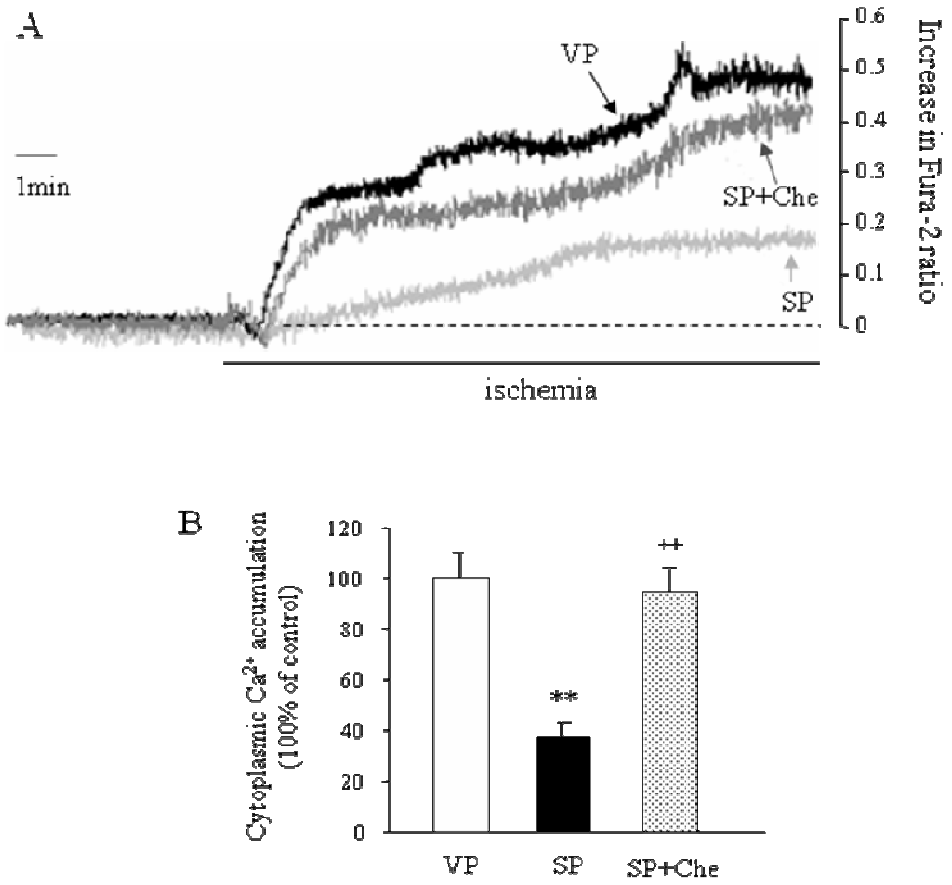

Figure 4-5. Effects of SP on cytosolic Ca^{2+} accumulation during ischemia in the presence and absence of PKC inhibitor. (A) Typical tracings of resting Ca^{2+} level during 15 min of ischemia challenge in single cardiomyocytes in VP, SP and SP+Chelerythrine (SP+Che). (B) Group results. Data are presented as the area under the Ca^{2+} increase curve within 10 min of ischemia and normalized to 100% of control. Values are mean ± SEM, n=6, $^{**}P<0.01$ vs VP, $^{++}P<0.01$ vs SP.

4.3.6 SP attenuated myocyte hypercontracture at the onset of reperfusion in a PKC-dependent manner

Hypercontracture refers to the sustained maximum contractile activation of myofibrils resulting from the combination of excessive cytosolic Ca^{2+} accumulation during ischemia and energy resupply after reperfusion (Siegmund et al., 1993). We determined cell length shortening few minutes after the onset of reperfusion in an attempt to investigate whether SP may also alleviate hypercontracture. As shown in Fig. 4-6, in VP group myocyte length was shortened to about half of their initial length after reperfusion. SP significantly attenuated this detrimental shortening and this effect was abolished by inhibition of PKC with chelerythrine.

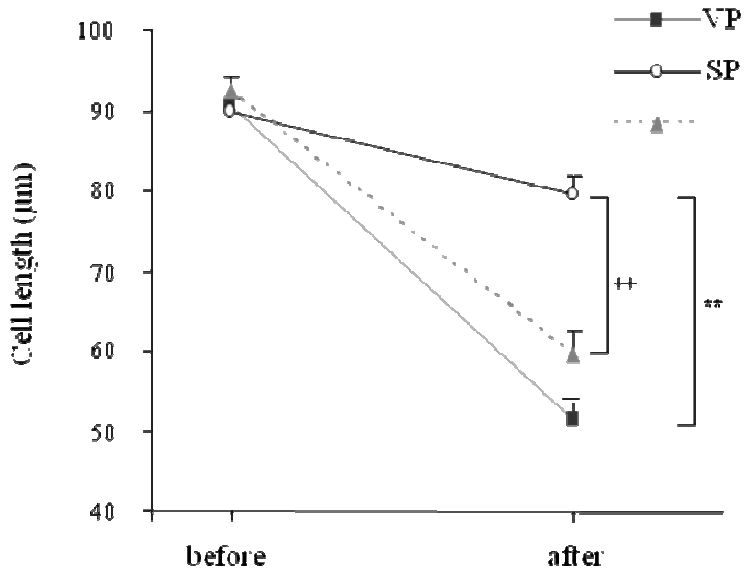

Figure 4-6. Effects of SP on myocyte hypercontracture at the onset of reperfusion. Mean data showing the cell lengths before ischemia and after onset of reperfusion in VP, SP and SP+Chelerythrine (SP+Che). Mean ± SEM, n=30~50, $^{**}P<0.01$ vs VP, $^{++}P<0.01$ vs SP.

4.4 Discussion

In the current study we found that SP-induced PKC isoform activation accelerates the rectification of elevated $[Ca^{2+}]_i$ and thereby increases the susceptibility of cardiomyocytes

to ischemia-induced Ca^{2+} overload and consequent damages induced by reperfusion. These findings disclose a novel mechanism for SP-induced cardioprotection and also provide considerable implication for other PKC-related anti-ischemia interventions.

4.4.1 PKC isoform translocation

Although PKC activation seems to be coupled with the genesis of late phase of cardioprotection induced by SP, the specific PKC isoforms involved is still unknown. PKC-α, ε, δ are the three main isoforms expressed in adult cardiomyocytes and also the most important ones involved in cardioprotection of ischemic preconditioning (Rybin and Steinberg, 1994; Kawamura et al., 1998). For this reason, we examined the effect of SP on these three isoforms before ischemia insults which itself can stimulate PKC translocation. We found that H_2S preconditioning motivated translocation of the three isoforms PKC-α, ε and δ to membrane fraction at 20h after preconditioning. Such translocation prior to ischemia attack may act as an essential step to switch the cells into a state tolerant to ischemia insults, and failure of such translocation results in the loss of cardioprotection as observed in the presence of a PKC inhibitor.

Individual PKC isozymes are believed to mediate characteristic cell functions, as upon stimuli they are directed to distinct subcellular membrane regions by isozyme-specific receptors for activated C kinase (RACK) (Mackay and Mochly-Rosen, 2001). By binding to their specific RACKs the activated isozymes are anchored close to their particular substrates. In the present study, we employed IP as a reference model due to its recognized stimulatory effect on PKC. Intriguingly, we found that IP only promoted PKCε and PKCδ translocation but not that of PKCα. The discrepancy between SP and IP suggests that they may employ different subsets of PKC isoforms to convey their

cardioprotective signals to different subcellular regions, affording probably similar but not identical cardioprotection.

4.4.2 PKC and K_{ATP}

Since H_2S has been shown to have a direct effect on K_{ATP} channels (Zhao et al., 2001; Tang et al., 2005), it raises the question whether SP-induced PKC activation is secondary to the opening of K_{ATP} channels. Unexpectedly, we observed that blockade of K_{ATP} channel only diminished the SP-induced translocation of PKCε but failed to affect the translocation of PKCα and δ. Thus, K_{ATP} channel opening may only be necessary for PKCε activation in the SP signaling pathway.

It is until recently that individual PKC isoforms were found located differently in relation to K_{ATP} channels in the cardioprotective signaling pathway. Hassouna demonstrated that PKCε is located upstream whereas PKCα is downstream to mitoK_{ATP} channel in IP signaling pathway (Hassouna et al., 2004). This implies a considerable diversity of the signaling mechanisms whereby PKC isoforms are activated. Since PKC can also be activated by other signaling molecules like NO or Ca^{2+} (Miyawaki and Ashraf, 1997; Ping et al., 1999), more studies are warranted to test whether SP induces activation of PKCα and PKCδ through provoking the release of these signaling molecules.

4.4.3 PKC and intracellular Ca^{2+} handling

Of great importance, we elucidated the mechanism how the SP-activated PKC mediates the cardioprotection. By monitoring the resting Ca^{2+} level in single cardiomyocytes, we observed that SP lowered elevation of $[Ca^{2+}]_i$ during ischemia in a PKC-dependant manner. Such a timely rectification on elevated $[Ca^{2+}]_i$ during ischemia challenge could

be therapeutically important, as uncontrolled elevation in $[Ca^{2+}]_i$ could induce irreversible injuries like mitochondria dysfunction (Minezaki et al., 1994), membrane degradation and contractile derangement (Gross et al., 1999). If the ischemia is followed by reperfusion, the myocytes will exhibit hypercontracture at the onset of reperfusion due to massive stimulation on the contractile machinery by accumulated Ca^{2+} (Siegmund et al., 1993). In perfused myocardium, this hypercontracture is manifested by contraction band necrosis (Ganote, 1983). Even if the necrotic myocardium can be replaced by scar tissues in a subsequent remodeling process, the akinetic fibrotic tissue will permanently impair the pumping function of the heart and when substantial enough will lead to heart failure (Richardson et al., 1996).

To further corroborate the effect of H_2S on resting Ca^{2+} during ischemia, we examined the myocyte hypercontracture at the onset of reperfusion. Indeed, SP reduced the development of myocyte hypercontracture through a PKC-dependent pathway. These beneficial effects triggered by SP and mediated by PKC could in turn at least partly account for the cardioprotection observed on cell viability, cell morphology, and cell function. It is also predictable that this limitation on development of Ca^{2+} overloading and hypercontracture in single cells would achieve further significant benefits by preserving contractile function in the intact heart.

Previous studies have demonstrated an effective approach to attenuate myocyte hypercontracture by increasing SERCA activity (Abdallah et al., 2005). Since SR uptake through SERCA presents the dominant route for Ca^{2+} removal in cardiomyocytes, it is plausible that this reduced hypercontracture is due to a faster Ca^{2+} clearing from cytosol before reperfusion. Enlightened by this finding, we examined the SR- Ca^{2+} uptake rate as

well as the minor mechanism for Ca^{2+} removal, i.e. extrusion via NCX. We found that SP accelerated the clearing rate through both of these routes. Again, all these beneficial effects induced by SP were reversed by inhibition of PKC, implying that PKC may phosphorylate these calcium handling proteins and improve their function.

In conclusion, the present study significantly advances our understanding on the SP-induced cardioprotection by delineating the essential role of PKC in the context of signaling pathway. The results demonstrate that SP activates PKCα, ε and δ in cardiomyocytes, among which only activation of PKCε is secondary to the K_{ATP} channel opening. Such PKC activation accelerates cytosolic Ca^{2+} clearing and prevents development of Ca^{2+} overloading and myocyte hypercontracture during ischemia and reperfusion.

Chapter 5 H_2S preconditioning induces late cardioprotection in a rat model of myocardial infarction

5.1 Introduction

Myocardial infarction (MI) is the common presentation of ischemic heart disease. To test the cardioprotective effect of H_2S in intact animals, we established a rat model of MI by occluding the left anterior descending coronary artery (LAD). To pave way for clinical studies, we investigated and fine-tuned the effect of H_2S preconditioning and further made comparison between different administration regimes to investigate whether post-MI treatment with H_2S could produce comparable infarct-limiting effect and whether a combination of both could provide additional protection.

5.2 Materials and Methods

5.2.1 Animals

The study protocol was approved by the Institutional Animal Care and Use Committees (IACUC) of National University of Singapore. Left ventricular (LV) MI was created in 7-week-old male Sprague-Dawley rats. The rat was anesthetized by an intraperitoneal injection of ketamine (70mg/kg body weight) and xylazine (4.6mg/kg body weight). A 3-cm catheter was inserted into the animal's trachea and the animal was ventilated with a Havard respirator at 85 strokes per minute and tidal volume of 2.15 cc. After thoracotomy at the fourth intercostal space, the heart was exteriorized and the left anterior descending coronary artery (LAD) was permanently ligated using 6-0 suture. The heart was then placed back to normal position and the chest was closed with 3-0 suture.

5.2.2 Experimental design

The protocols were shown in Fig. 5-1. Rats in H_2S preconditioning group received a single bolus of NaHS (a H_2S donor) one day before surgery. NaHS was given intraperitoneally at doses of 0.1, 1, 3, 10, 30 μmol/kg body weight. Rats in H_2S post-MI treatment group received an intraperitoneal bolus of NaHS immediately after surgery and once a day for two more days. NaHS was given at 0.1, 1, 10 μmol/kg body weight. Rats in the combo group were treated with a combination of preconditioning and post-MI treatment, that is, a bolus of NaHS one day before surgery plus one bolus a day for 3 days after surgery. Each group was compared with correspondent vehicle-treated MI, in which rats received surgery and saline injection in parallel with NaHS administration. For the time course study (Fig. 5-3A), rats received surgeries 1 day, 3 days or 5 days after NaHS administration (1μmol/kg).

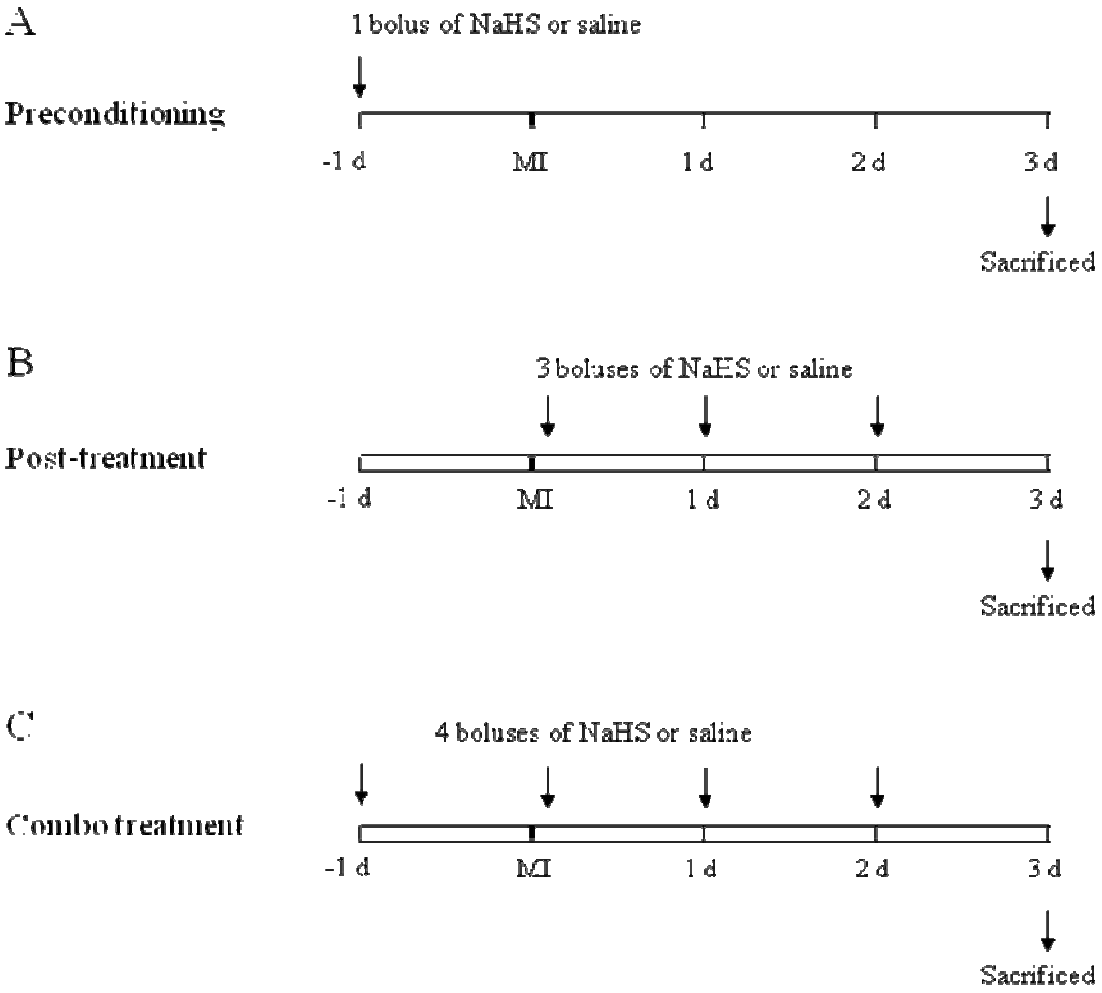

Figure 5-1. Experimental protocols. (A) H_2S preconditioning group: rats received a single bolus of NaHS (a H_2S donor) one day before surgery; (B) H_2S post-treatment group: rats received an intraperitoneal bolus of NaHS immediately after surgery and once daily for two more days; (C) Combo treatment group: rats were treated with a combination of H_2S preconditioning and post-MI treatment, namely, a bolus of NaHS given once daily from one day before till 3 days after surgery.

5.2.3 Assessment of infarct size

Infarct size and ischemic risk area were determined using Evans blue and triphenyltetrazolium chloride (TTC) staining. Three days after MI, animals were euthanized, the heart excised, and stained with 0.12% evans blue to define the area at risk (AAR; the nonperfused and hence unstained myocardium). The heart was then sliced into

sections and incubated in 1% TTC in PBS for 15 minutes to define the area of infarction (INF; nonviable thus unstained myocardium). Infarct size, ventricle internal diameter and anterior wall thickness were assessed using computerized planimetry.

5.2.4 Statistics

All data are expressed as a mean ± SEM. Statistics were performed with one-way ANOVA followed by a Bonferroni post-hoc test. A *P* value less than 0.05 denotes a statistically significant difference.

5.3 Results

5.3.1 AAR/LV was consistent throughout the study

Table 1 lists the number of animals in each group and the correspondent ratio of area at risk to total left ventricle area (AAR/LV). No significant difference in AAR/LV was observed across groups.

Table 1. List of experimental groups with their respective animal numbers and area at risk, expressed as the percentage of the left ventricle (AAR/LV).

Study	Group	N	AAR/LV (%)
Preconditioning	MI-vehicle	7	52.6 ± 2.8
	NaHS Pre 0.1	7	50.6 ± 3.0
	NaHS Pre 1	8	50.5 ± 4.5
	NaHS Pre 3	7	52.9 ± 4.0
	NaHS Pre 10	7	52.3 ± 1.7

	NaHS Pre 30	6	48.0 ± 2.9
Post-treatment	MI-vehicle	9	51.8 ± 3.3
	NaHS Post 0.1	7	49.6 ± 2.5
	NaHS Post 1	7	50.2 ± 2.9
	NaHS Post 10	8	50.8 ± 3.8
Combo treatment	MI-vehicle	8	50.4 ± 2.5
	NaHS Combo 0.1	7	51.2 ± 3.2
	NaHS Combo 1	7	48.8 ± 3.6
	NaHS Combo 10	10	52.4 ± 3.9
Time course study	MI-vehicle (1 day)	6	50.9 ± 2.5
	NaHS pre 1 (1 day)	6	49.4 ± 4.2
	MI-vehicle (3 day)	7	53.4 ± 3.0
	NaHS pre 1 (3 day)	6	50.7 ± 3.8
	MI-vehicle (5 day)	6	51.3 ± 3.9
	NaHS pre 1 (5 day)	5	49.7 ± 2.7

5.3.2 H_2S preconditioning reduced infarct size, LV dilatation and wall thinning in the heart undergoing MI

A single bolus of NaHS was administered one day before MI (Fig. 5-1A). As shown in Fig. 5-2B, NaHS at 0.1, 1, 3, and 10μmol/kg significantly decreased infarct size per AAR as compared with MI-vehicle group. The optimal effect was found in rats receiving 1 μmol/kg NaHS, which displayed a 78% reduction in infarct size. Representative mid-ventricular cross sections of MI-vehicle and H_2S preconditioning at 1 μmol/kg were shown in Fig. 5-2A. H_2S preconditioning also remarkably reduced LV dilatation and wall thinning, as manifested by the decreased LV internal diameter (Fig. 5-2C) and increased anterior wall thickness (Fig. 5-2D) compared with MI-vehicle. Similar dose-response curves were observed, with significant effects found within a dose range of 0.1~3 μmol/kg NaHS.

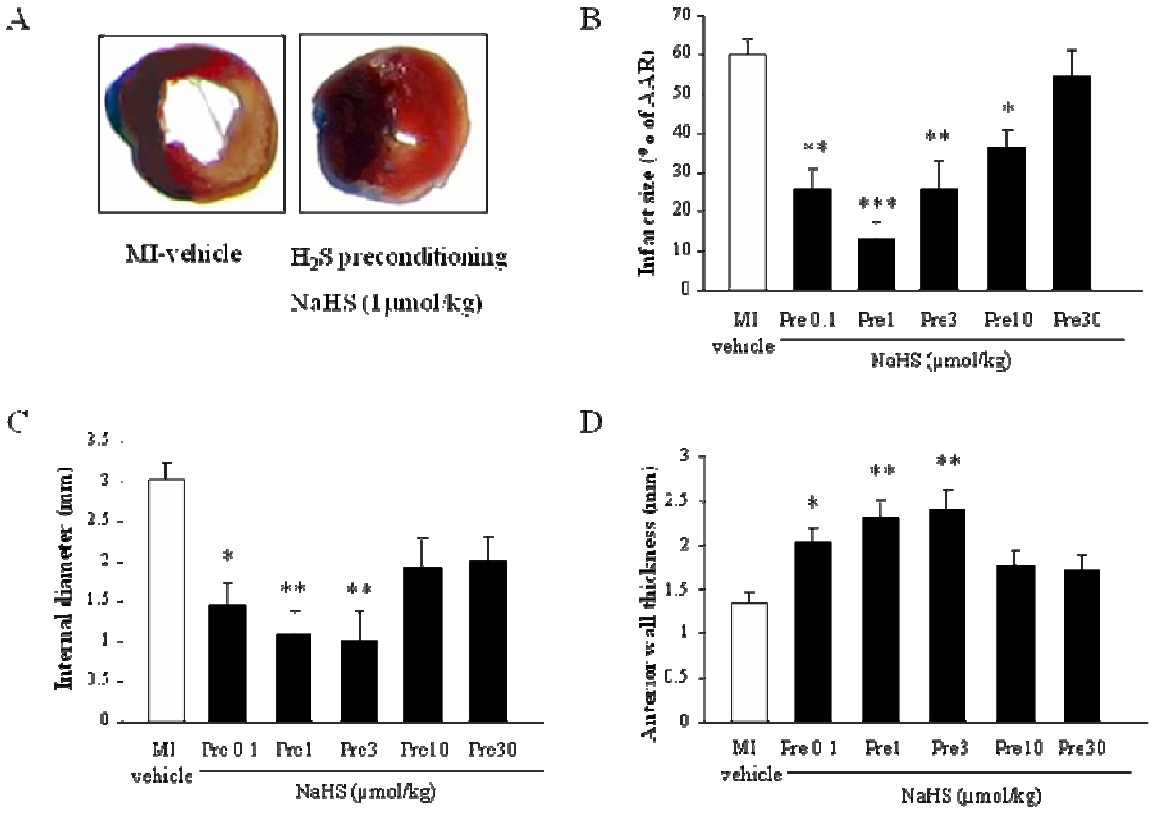

Figure 5-2. Effect of H_2S preconditioning on infarct size and LV geometry. (A) Representative mid-myocardial sections of MI-vehicle and H_2S preconditioning. (B-D) Dose-dependent effect of NaHS (0.1~30 μmol/kg) on infarct size (B), left ventricle internal diameter (C), and left ventricle anterior wall thickness (D). The data are presented as mean ± SEM. *, P<0.05, **, P<0.01, ***, P<0.001vs. MI-vehicle. N per group is shown in Table 1.

5.3.3 The protection of H_2S preconditioning lasted at least 3 days after NaHS administration

Rats preconditioned with NaHS (1 μmol/kg) were subjected to coronary occlusion on day 1, day 3 or day 5 after NaHS administration (Fig. 5-3A). Strong infarct-limiting effects were observed on day 1 and day 3 (Fig. 5-3B), indicating that the protection lasted at least 3 days after the preconditioning stimulus.

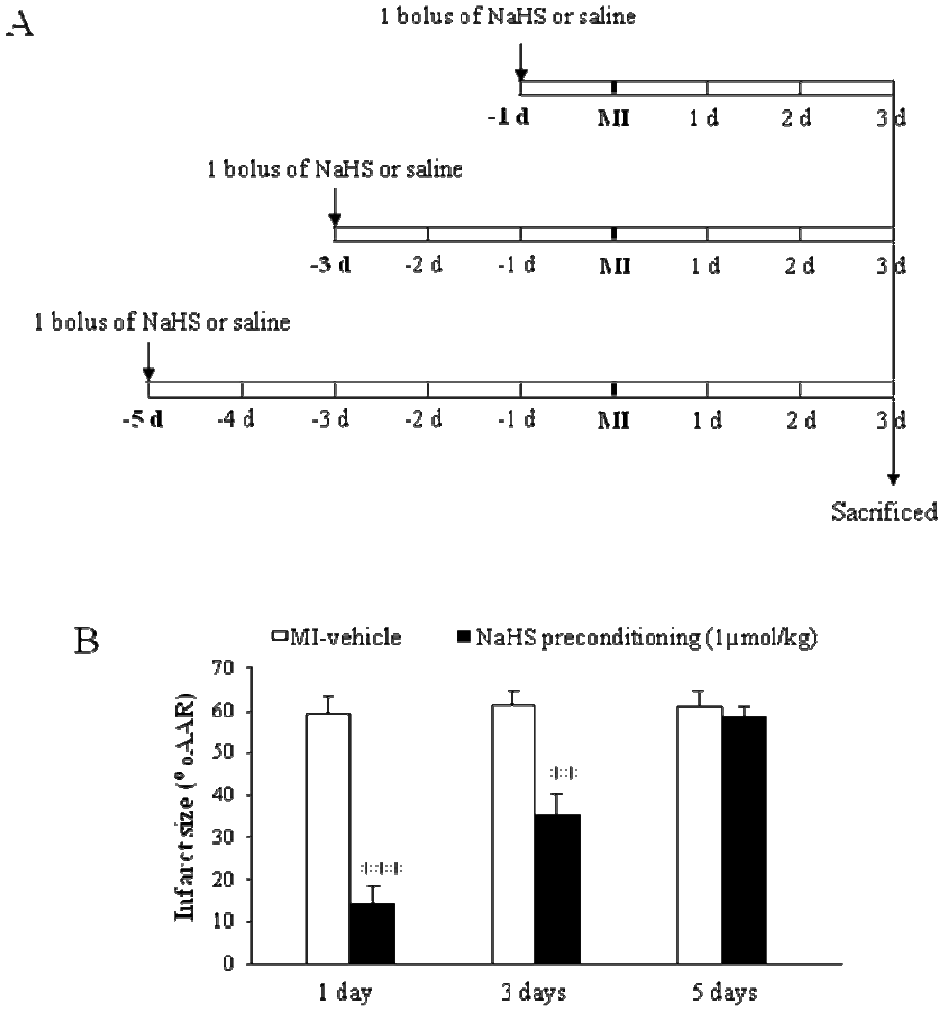

Figure 5-3. Time course of the cardioprotection induced by H_2S preconditioning. (A) Experimental protocol. (B) Infarct size in rats subjected to MI on day 1, day 3 and day 5. The data are presented as mean ± SEM. **, P<0.01, ***, P<0.001 vs. correspondent MI-vehicle. N per group is shown in Table 1.

5.3.4 The protection of H_2S preconditioning could not be replaced with H_2S post-MI treatment

Based on the effective dose range of H_2S preconditioning, we examined the effect of H_2S post-treatment at 0.1, 1, and 10 µmol/kg NaHS given once daily for 3 days after the MI surgery (Fig. 5-1B). Rats receiving 1 and 10 µmol/kg NaHS also displayed a significant decrease in infarct size compared with correspondent MI-vehicle group (Fig. 5-4A). However, when compared with H_2S preconditioning group at the same dose, the infarct/AAR was significantly lower in preconditioning groups than those in post-treatment groups. At the optimal dose of 1 µmol/kg NaHS for both groups, H_2S preconditioning limited the infarct size to 13.1 ± 4.3% per AAR, representing a 78% reduction, while H_2S post-treatment reduced the infarct size only by 38% to 37.5 ± 3.1% per AAR. In addition, none of the doses in post-treatment group produced significant effect on LV dilatation and wall thinning, as indicated by LV internal diameter (Fig. 5-4B) and wall thickness (Fig. 5-4C). In contrast, H_2S preconditioning significantly decreased LV internal diameter and increased wall thickness when compared with either MI-vehicle group or H_2S post-treatment at 1 µmol/kg.

We also tested whether a combination of H_2S preconditioning and post-MI treatment could produce a synergic cardioprotection. Rats in the combo group received NaHS (0.1, 1, or 10 µmol/kg) injection once daily from 1 day before till 3 days after MI surgery (Fig. 5-1C). As shown in Fig. 5-4A, a combo treatment of NaHS at all doses significantly decreased infarct size compared with correspondent MI-vehicle group. When comparing at the same dose collaterally, the infarct/AAR in combo group at 0.1 and 1 µmol/kg were significantly lower than that in post-treatment group but comparable to that in H_2S

preconditioning group. Likewise, a similar trend was observed when assessing the LV parameters (Fig. 5-4 B&C). In addition, no significant difference in infarct size and LV geometry was observed across the three MI-vehicle groups injected with the three different administration regimes. These results indicate that the infarct-limiting effect of H_2S preconditioning superseded that produced by post-treatment when both were administered. Continuous treatment after MI did not reinforce the effect of H_2S preconditioning and thus seems redundant.

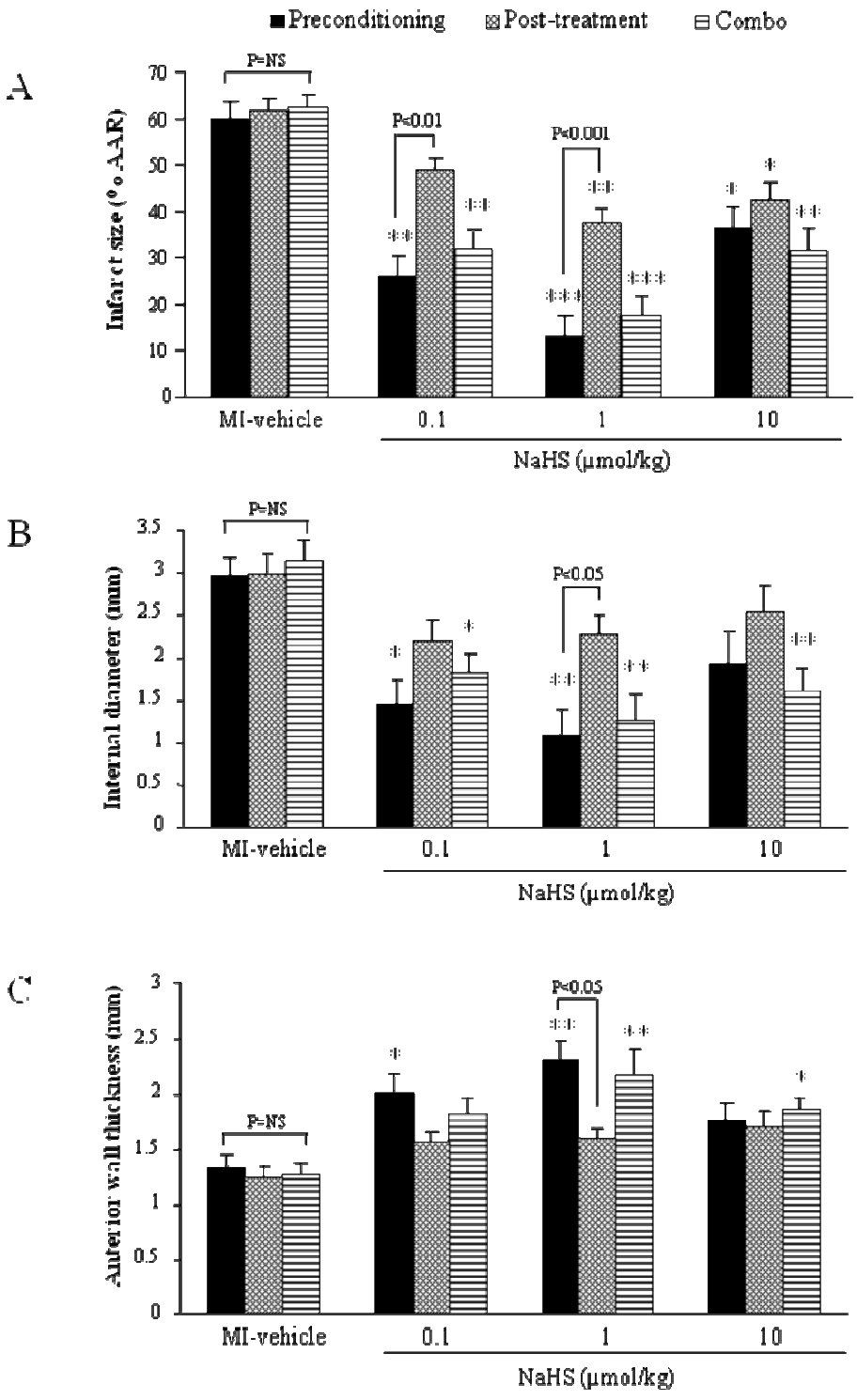

Figure 5-4. Comparison on infarct size (A) and LV internal diameter (B) and LV anterior wall thickness (C) among H_2S preconditioning, post-MI treatment and a combination of both (combo). NaHS was given at 0.1, 1, 10 μmol/kg for each group. The experimental protocol is shown in Figure 1. The data are presented as mean ± SEM. *, $P<0.05$, **, $P<0.01$, ***, $P<0.001$ vs. correspondent MI-vehicle. N per group is shown in Table 1.

5.4 Discussion

The present study demonstrated in an *in vivo* rat model that brief exposure to H_2S restrains the extent of myocardial infarction that occurs in the next 72 hours through a PKC-dependent mechanism. Post-MI treatment with NaHS at the same doses for 3 days did not produce as pronounced infarct-limiting effect as observed in the preconditioning group. A combination of both did not produce additional benefit more than preconditioning alone. These results suggest that the effect of preconditioning is not replaceable with post-MI treatment even with multiple-administrations. The access to preconditioning, by and large, determines the outcome of the patients. Continuous treatment after MI is not necessary when the access to preconditioning has been secured.

As always, studies in intact animals are of great importance to bridge the novel discoveries in basic research to clinical use. Since H_2S has been proposed to be both cytoprotective (Kimura and Kimura, 2004; Whiteman et al., 2004; Whiteman et al, 2005) and cytotoxic (Deplancke and Gaskins, 2003; Yang et al., 2006), depending on the cell type and concentration used, fine-tuning the NaHS dose is particularly crucial in *in vivo* experiments. Inappropriate selection of dose range may lead to misappraisal of its therapeutic value or even to contradictory results. The prevailing dose of sulfide donor used in the most of recent cardiovascular studies is above 10 μmol/kg body weight per day (Cai et al., 2007; Meng et al., 2007; Sivarajah et al., 2007; Zhu et al., 2007). However, we demonstrated in a preconditioning model that a single bolus of NaHS at 0.1~1μmol /kg was sufficient to afford marked protection against MI. Increasing the dose from 1μmol /kg only decreased the protection.

We also performed a time course study to determine the duration of the protection. Sivarajah and colleagues (Sivarajah et al., 2007) showed that NaHS pretreatment, 15 min before MI, produced immediate protection, while we found that H_2S preconditioning produced late protection which lasted at least 3 days. The vastly different duration of protection supports the concept that the late phase of preconditioning may have greater clinical usefulness (Bolli, 2000). It also merits further study to investigate whether continuous administration of NaHS at 48-72h intervals will extend the protection duration and whether repeated administration will result in the maintenance of a defensive phenotype.

Although preconditioning is inferior to treatment in terms of practical convenience, the ultimate goal should be identification and implement of the most effective approach to intervene with MI. Elrod and colleagues recently reported that H_2S donor administered at the time of reperfusion significantly decreased infarct size (Elrod et al., 2007). However, no comparison has so far been conducted between the effectiveness of H_2S preconditioning and post-MI treatment. Our current study evidenced that H_2S preconditioning produced far stronger infarct-limiting effects than H_2S post-treatment in this model, indicating that the effect of preconditioning with a single bolus of NaHS could not be replaced with multiple post-MI administration of the sulfide donor. This result bears great clinical implication for those potential patients at high risk of myocardial infarction. Brief exposure to low dose of H_2S may make them survive a MI which would otherwise be a lethal attack. However, much less benefit could be provided by H_2S treatment after the attack has occurred. To substantiate above finding, we treated the rats with a combination of both preconditioning and post-MI treatment. The

combined treatment produced an infarct-sparing effect comparable to that produced by H_2S preconditioning alone, which further underscores the importance of access to NaHS before the attack.

In the current study, we also examined the effect of H_2S on LV chamber dilatation and wall thinning by assessing the LV internal diameter and anterior wall thickness. Changes in these parameters almost followed the same trend as infarct size. It is worthy of note that H_2S post-treatment did not reduce LV dilatation and wall thinning to a statistically significant extent in spite of deceasing infarct size, while preconditioning was able to effectively limit infarction, chamber dilatation and ventricle wall thinning at the same time. These effects of H_2S preconditioning on ventricular geometry hint a better preserved LV pump function and a reduced chance of post-MI ventricular rupture.

With accumulating data supporting its protective effect, H_2S possesses the potential to be developed into an inhaled gas or a parenteral injectable, which could actualize the power of ischemic preconditioning without feasibility problem. Importantly, the dose of H_2S we identified most effective in the preconditioning model is over 250 folds lower than its LD_{50} value (Warenycia et al., 1989). Therefore, for the high-risk population, brief exposure to H_2S every 3 days represents a cost-effective prevention measure.

In conclusion, the current study provides the first evidence that 1) preconditioning with low concentration of H_2S produces delayed cardioprotection against myocardial infarction and 2) H_2S preconditioning was far more effective than post-treatment in limiting infarct size, LV dilatation and wall thinning.

Chapter 6 General discussion

The whole investigation profiled the role of hydrogen sulfide in the cardioprotection against ischemia and reperfusion insult, focusing on its potential as a preconditioning agent. The evidence from both *in vitro* and *in vivo* studies consistently pointing towards that H_2S preconditioning produces potent cardioprotective effects, which translated into decreased cell death, prevention of intracellular calcium overload, preserved contractile function and restraint of infarct size. The dose-response curve, both in isolated cardiomyocytes and in intact animal, is bell-shaped: raising the dose of sulfide above the optimal dose results in diminished therapeutic efficacy.

Using cardiomyocytes, we also identified several essential signaling components and intracellular events underlying the protection afforded by H_2S preconditioning. Fig. 6-1 summarizes the current understanding of H_2S preconditioning by proposing a signaling pathway: with free passage through the plasma membrane, H_2S directly opens sarcolemmal K_{ATP} channel which activates PKCε. Or through some unknown mechanisms, H_2S could also directly or indirectly stimulate PKCα and PKCδ. Activated PKC isoforms then translocate from cytosol to membrane fraction of the cell, where calcium handling proteins such as SERCA and NCX are located. PKC may enhance the function of these proteins whereby the cytosolic calcium clearing is accelerated. During ischemia and reperfusion, fast calcium clearing results in attenuated calcium accumulation in the cytosol and reduced hypercontracture of the myocytes, both of which contribute to the decrease in infarct size and preservation of contractile function.

However, we also bear in mind that the whole scenario of the signal transduction could be much more complicated. The signaling pathway outlined above is by no means the only goings-on after H_2S preconditioning. Taking PKC as a nodal point, a spectrum of endogenous molecules, like NO, adenosine, and free radicals, could be its upstream triggers, while its downstream targets could range from mitogen-activated protein kinases, heat shock proteins to mitochondria proteins (Sanada and Kitakaze, 2004). It is more likely to be a signal network than single pathway which transforms the extracellular stimulus of H_2S into the final protection.

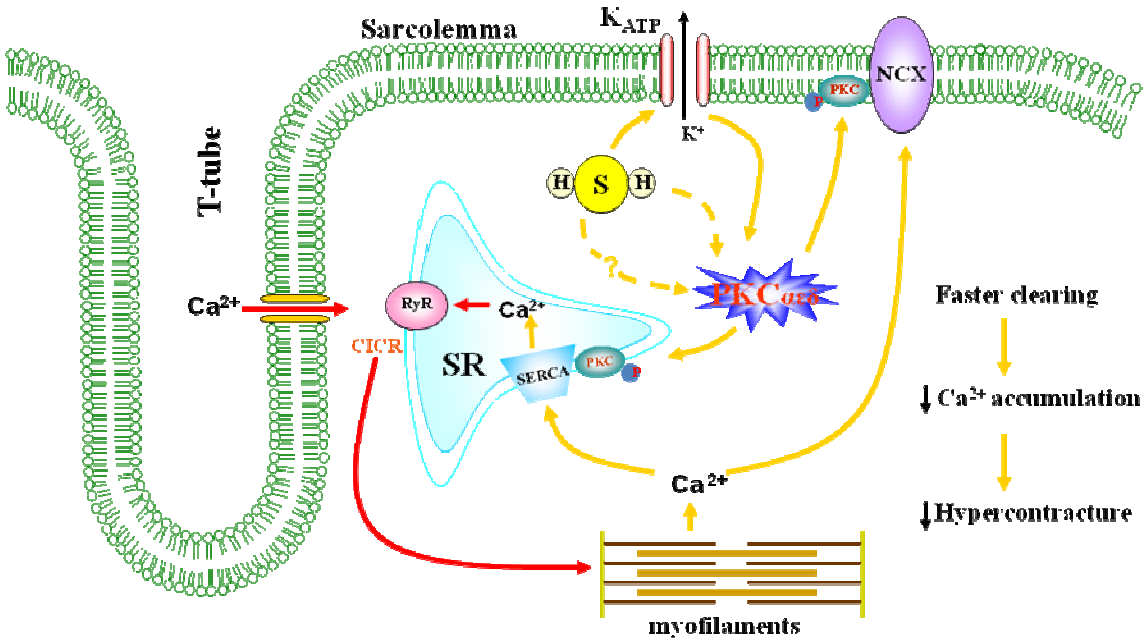

Figure 6-1. The proposed signaling pathway for SP-induced cardioprotection (the yellow route). H_2S activates different PKC isoforms directly (dashed line) or indirectly though the opening of K_{ATP} or other unknown mechanisms (dashed line). The activated PKC isoforms stimulate the Ca^{2+} handling proteins (i.e. NCX and SERCA) and thereby facilitate the clearing of cytosolic Ca^{2+}. During ischemia, the faster clearing of cytosolic Ca^{2+} induced by SP attenuates the Ca^{2+} accumulation and reduces hypercontracture.

In the present study, we observed three PKC isoforms activated by H_2S preconditioning, but the inhibitor chelerythrine could not distinguish the one or ones that is necessary for the genesis of the late cardioprotection. It is likely that different isoforms act on different substrates at various subcellular sites and afford the protection from diverse aspects (Mackay and Mochly-Rosen, 1999). Assigning specific roles to each PKC isoform may depend on the availability of isoform-specific antagonists, siRNA or PKC-isoform knockout animals.

Using intact animal model, we also demonstrated that H_2S preconditioning could provide much stronger protection than H_2S post-treatment. This finding prompts us to reason that the mechanisms underlying preconditioning and post-treatment are different. H_2S preconditioning is more likely to protect by switching the heart to a defensive state against ischemia insult. The opening of K_{ATP} channels, activation of PKC isoforms as well as altered intracellular calcium handling are accomplished prior to the attack. Thus even though exogenous H_2S was not present at the time of attack, the protection was still well executed. In contrast, without H_2S preconditioning, the endogenous defensive system could be overwhelmed by the injuries caused by lethal ischemia, and supplement with exogenous H_2S after the attack could only provide limited benefit. The effect of H_2S -posttreatment may only rely on the ability of sulfide to reduce inflammatory responses (Zanardo et al., 2006) and to neutralize cyotoxic reactive species such as peroxynitrite (Whiteman et al., 2004), which may relieve the oxidative stress to some extent but not likely to reverse infract myocardiums back to normal.

Another important question is that the local H_2S concentration achieved using sulfide donor is unknown. The basal H_2S level in the rat serum was reported to be ~46μM and

physiological range was assumed to be within 50~150 μM, depending on the tissue of interest (Wang, 2002). However, in our *in vivo* study, the dose of H_2S that produced maximum protection was at 1μmol/kg, which may increase the circulating H_2S by 10~20μM (the blood volume of a 250g rat is ~15.5ml) (Lee and Blaufox, 1985). If there is a substantial background level of sulfide, how is it possible that relatively small increment can produce so significant biological effects? One possibility is that the biological sulfide-eliminating systems might get saturated by sudden exposure to sulfide, and the non-metabolized sulfide locally in the heart triggers the downstream biological effects.

However, evidence is accumulating in favor of another possibility—the exact free sulfide baseline levels in blood and tissues are probably lower than the reported levels. Although we do not question the detection of endogenous H_2S, we must note that the reported baseline concentration of H_2S is high enough to emit the characteristic unpleasant H_2S smell, whereas the blood samples actually do not. In two recent reviews by Li (Li and Moore, 2008) and Szabó (Szabó, 2007), both authors mentioned that the colorimetric assays or ion selective electrode assays used to measure H_2S concentrations in most studies are likely to liberate sulfide from its bound forms, thereby generating concentrations that are likely to represent a mixture of free and bound sulfide (Hannestad et al., 1989; Togawa et al., 1992; Ogasawara et al., 1993). Using a lately-developed polarographic H_2S sensor, Whitfield and colleagues have recently reappraised sulfide concentration in vertebrate blood (Whitfield et al., 2008). They found that H_2S gas was undetectable (<100nM) in blood from numerous animals, including lamprey, trout, mouse, rat, pig and cow. Interestingly, exogenous H_2S was also rapidly removed from

blood or plasma. Indeed, as a highly reactive molecule, H_2S could either be broken down rapidly by enzymes, sequestered by binding to haemoglobin, or react chemically with a number of species abundant in tissues, including superoxide radical, hydrogen peroxide, peroxynitrite and/or hypochlorite (Li and Moore, 2008). The key issue that needs to be addressed in future studies is how the fleeting presence of free sulfide achieved subsequent lasting biological effects.

To date, simple sulfide salts, most commonly NaHS, have been the H_2S-releasing drugs used in most biological experiments, including our present study. NaHS is known to release H_2S instantaneously in aqueous solution. Because the release of endogenous H_2S from cells is likely to occur in lesser amounts and at a much slower rate, NaHS may not mimic the biological effects of naturally produced H_2S. However, based on findings in the current study, NaHS is able to produce both immediate and delayed protection against cardiac ischemia, even if it only transiently increases the circulating H_2S levels. Thus for the preconditioning purpose, NaHS qualifies as an economical option. Development of organic compounds that slowly release free H_2S over extended periods of time could be more useful for treating patients who fail to access to H_2S preconditioning before myocardial infarction occurs or treating other diseases where long period of H_2S treatment has shown therapeutic effects.

Another potential area is the development of H_2S as an inhaled gas or as a parenteral injectable. Inhaled NO has set a precedent for development of medical gases. NO was also first known as a toxic gas and is currently approved for use in infants with primary pulmonary hypertension (Kinsella and Abman, 2005; Hillier, 2003). However, the unpleasant odor of H_2S may pose more problems for administration, which could

necessitate the implementation of appropriate trapping systems to prevent from spreading into environment and exposure of medical personnel (Szabó, 2007). With enteral or parenteral formulations, odor would not create a problem, but manufacturing and formulation issues remains challenging compared with conventional chemical compound. In addition, animal safety data are still lacking with respect to parenteral or enteral administration of H_2S. Although sulfide is an endogenous substance, all exogenous sulfide delivery systems would be required to pass stringent safety and efficacy tests in preclinical animal studies before progression into human studies.

Chapter 7 Conclusion

The current study demonstrated that H_2S is both necessary and sufficient for the development of early and late phases of cardioprotection against ischemia. Cells or animals preconditioned with H_2S displays a phenotype tolerant to experimental ischemia or myocardial infarction, manifested by the decreased cell death or infarct size. Such protection is mediated by sarcolemmal K_{ATP} channels, NO and PKC. Activation of PKC results in accelerated cytosolic Ca^{2+} clearing, which prevents development of Ca^{2+} overloading and myocyte hypercontracture during ischemia/reperfusion insult. These findings demonstrate the potential of H_2S preconditioning as an effective intervention approach against ischemic heart disease. For the high-risk population, H_2S preconditioning represents a realistic and cost-effective prevention measurement.

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