

**Roles and mechanisms of action of the
L-cysteine/cystathionine- γ -lyase/hydrogen
sulphide pathway in the heart**

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Abstract

Hydrogen sulphide (H_2S) is a naturally occurring gas and originally the primary focus of research was to investigate its toxicity. In 1989 a physiological role of H_2S was proposed after endogenous levels were detected in the rat brain and normal human post-mortem tissue. This discovery has led to an explosion of interest in H_2S as a biological mediator. Identification of H_2S synthesising enzymes in the cardiovascular system has led to a number of studies examining specific regulatory actions of H_2S . The hypothesis underlying the studies in this thesis was that H_2S synthesising enzymes exist in the myocardium and the resulting H_2S provides cardioprotection against ischaemia-reperfusion injury. This was investigated using a broad range of experimental techniques including Langendorff isolated perfused rat heart models, biochemical H_2S stimulation and detection assays, PCR, and Western blotting. The principal findings can be summarised as follows:

1. Rat myocardium has the potential to express both CSE and CBS H_2S synthesising enzymes, due to the confirmed detection of mRNA.
2. Furthermore it was possible to exogenously stimulate the CSE enzyme, with its substrate L-cysteine, to produce H_2S gas which limited infarct size during regional ischaemia-reperfusion.
3. Endogenous H_2S levels were up-regulated during ischaemia-reperfusion, consistent with an endogenous protective role within the myocardium.
4. Simple and complex H_2S /thiol containing compounds produced cardioprotection during regional ischaemia-reperfusion, with a mechanism that involves PI3k and Akt activation, implicating recruitment of downstream kinases within the RISK pathway.

The studies presented have provided a significant advancement in understanding the involvement of H_2S in cardioprotection during ischaemia-reperfusion. It has also raised questions such as the exact mechanism of action of H_2S donor/thiol containing compounds and highlighted the need for more robust H_2S donors. The scope for H_2S as an endogenous mediator also stems beyond that of cardioprotection, as the range of body systems and cell types are continually expanding.

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Abbreviations

'	Minutes
"	Seconds
Abs	Absorbance value
Akt	Protein kinase B
ANOVA	Analysis of variance
ANP	Atrial natriuretic peptide
ANT	Adenine nucleotide translocator
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BNP	B-type natriuretic peptide
BSA	Bovine serum albumin
CAO	Coronary artery occlusion
cDNA	Complementary deoxyribonucleic acid
CFR	Coronary flow rate
cGMP	Cyclic guanosine 3',5'-monophosphate
CO	Carbon monoxide
CYP-D	Cyclophilin-D
DMSO	Dimethyl sulphoxide
ECL	Enhanced chemiluminescence
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal regulated kinase
FAC	Final assay concentration
g	Relative centrifugal force
G-Ish.	Global ischaemia
GSK-3 β	Glycogen synthase kinase 3 beta
HR	Heart rate
HRP	Horse radish peroxidase
H ₂ S	Hydrogen sulphide
IGF1	Insulin-like growth factor 1
IP3	Inositol-1,4,5-triphosphate
IPC	Ischaemic preconditioning
IPOST	Ischaemic postconditioning
K _{ATP}	Adenosine triphosphate-sensitive potassium channel
L-NAME	N $_{\omega}$ -Nitro-L-arginine methyl ester
LV	Left ventricle
LVEDP	Left ventricular end-diastolic pressure
MAPK	Mitogen activated protein kinase
MESNA	2-mercaptoethane sulphonate sodium
mmHg	Millimeters of mercury
mPTP	Mitochondrial permeability transition pore
mRNA	Messenger ribonucleic acid
NaHS	Sodium hydrosulphide

NO	Nitric oxide
NOS	Nitric oxide synthase
NSAID	Non-steroidal anti-inflammatory drug
P-5'-P	Pyridoxal-5'-phosphate
p70s6K	Phosphoprotein 70 kDa ribosomal protein S6 kinase
PDK-1	3-phosphoinositide-dependent kinase 1
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol 3,4-bisphosphate
PIP3	Phosphatidylinositol 3,4,5-triphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
PVDF	Polyvinylidene difluoride membrane
ROCK	Rho kinase
ROS	Reactive oxygen species
PBS	Phosphate-buffered saline
PTEN	Phosphatase and tensin homolog
RPP	Rate pressure product
SDS	Sodium dodecyl sulphate
sGC	Soluble guanylate cyclase
SR	Sarcoplasmic reticulum
TBST	Tris-buffered saline
TCA	Trichloroacetic acid
SEM	Standard error of the mean
W/V	Weight by volume
VDAC	Voltage dependent anion channel

Chapter 1 General introduction

Chapter 1 Part I

1.1 Coronary heart disease

Cardiovascular disease accounts for 198,000 deaths per year in the UK and is the leading cause of premature death. Coronary heart disease and stroke are the main forms of cardiovascular disease, accounting for 48% and 28% of cardiovascular deaths respectively (Allender *et al.*, 2008). An additional 31,000 deaths occur from other forms of heart disease. In 2006 there were 126,000 deaths from heart disease in the UK. Death rates for coronary heart disease have been falling in the UK since the late 1970s, and for people under the age of 75, they have fallen by 40% in the last 10 years (Allender *et al.*, 2008). However, the rates in the UK are still amongst the highest in Western Europe. In the UK currently 2.7 million people have coronary artery disease (Allender *et al.*, 2008).

Cardiovascular disease is a global burden on healthcare resources worldwide and results in 15 million deaths per year. Coronary heart disease is also the leading cause of death worldwide, with the death of 3.8 million men and 3.4 million women each year (Yellon *et al.*, 2007). Current findings from World Health Organisation predict that as far as 2030, cardiovascular disease will remain the leading cause of mortality in the world (Mathers *et al.*, 2006).

The economic burden of coronary heart disease in the UK is very high, with the cost of healthcare at over £1.7 billion a year. The majority of the costs of coronary artery disease fall outside of healthcare and result from illness and death in those of working age and the economic effects on families who are caring for them (BHF, 2006). Whilst significant medical advances have been made in trying to reduce the mortality rates from coronary heart disease, new strategies and drug targets are still required.

An acute myocardial infarction (AMI), commonly referred to as a heart attack, is the single largest cause of mortality from coronary artery disease and accounts for 17 million deaths worldwide (Reeve *et al.*, 2005). An AMI occurs when there is a sudden impairment of the oxygen rich blood supply to a portion of the heart relative to its

metabolic demands, resulting in muscle death and subsequent heart failure. The most common cause of an AMI is the rupture of an unstable atheroma plaque resulting from coronary artery disease, which impairs the blood flow to the heart via the coronary artery (Kumar *et al.*, 2002). The mainstay of current therapeutic intervention is the restoration of blood flow to the heart, termed reperfusion, as rapidly as possible by removal of the blockage. The most commonly used techniques are thrombolytic therapy or primary percutaneous intervention (Yellon *et al.*, 2007). Unfortunately, as will be discussed in detail later, restoration of blood flow to the heart also carries a paradoxical risk of producing further damage (Yellon *et al.*, 2007). The rate of early death following an acute myocardial infarction is 10%, and the incidence of cardiac failure is 25% (Keeley *et al.*, 2003). More recently, greater emphasis is being placed on trying to prevent the occurrence of AMI through lifestyle changes such as encouraging diets lower in saturated fats and more active lifestyles (Hu *et al.*, 2002). Until such preventative measures are widely accepted into society, therapeutic interventions to limit the damage caused by AMI and the restoration of blood flow are required. Due to the worldwide incidence of AMI, research in this field is rapidly expanding through the use of experimental animal models to identify new targets which can be exploited at the time of reperfusion to limit the extent of further damage.

1.2 Myocardial infarction

1.2.1 Coronary artery disease

As mentioned previously an AMI most commonly occurs due to the rupture of unstable atherosclerotic plaques formed in coronary arteries (Kumar *et al.*, 2002). Coronary atherosclerosis arises from a complex interplay of inflammatory process caused by the accumulation of lipid, macrophages, and smooth muscle in the intimal plaques of large and medium sized coronary arteries. Atherosclerotic lesions are asymmetrical thickenings of the intima, the innermost layer of the artery. They are composed of cells, connective tissue, lipids, and debris (Stary *et al.*, 1995). Blood-borne inflammatory and immune cells compose an important part of the atheroma, with the remainder being composed of vascular endothelial and smooth muscle cells.

An accumulation of lipid-laden cells, mainly comprised of macrophages and T-cells, beneath the endothelium transforms the atheroma into a fatty streak (Stary *et al.*, 1994). The centre of the atheroma is composed of foam cells and extracellular lipid droplets which form a core region. This is surrounded by a cap of smooth muscle cells and a collagen rich matrix. Infiltration of T-cells, macrophages, and mast cells into the lesion occurs in the shoulder region where the atheroma expands (Stary *et al.*, 1995). Activation and production of inflammatory cytokines has been observed in the immune cells (Kovanen *et al.*, 1995). Ruptures of the plaque occur when the fibrous cap of the plaque is thin and partly destroyed (Falk *et al.*, 1995). The abundance of activated immune cells results in the production of inflammatory mediators and proteolytic enzymes that weaken the cap and activate the cells in the core (van der Wal *et al.*, 1994). This creates an unstable plaque that is liable to rupture, inducing a thrombus and resulting in an AML.

1.2.2 Mechanisms of myocardial injury

Relative to its oxygen needs, the heart is one of the most poorly perfused tissues in the body (Giordano, 2005). The heart is unable to extract oxygen and nutrients from blood within the atria and ventricles, and therefore relies on the coronary arteries as its own blood supply (Tune *et al.*, 2004). Under normal aerobic conditions the majority of cardiac energy is derived from fatty acids which provides between 60 – 90% of the energy for ATP synthesis (Bing, 1955). Carbohydrate metabolism only accounts for 10-40% of the energy generated in a healthy adult human heart at rest (Bing, 1955). A defining characteristic of ischaemia is that the oxygen supply is insufficient to support oxidative phosphorylation (Hearse, 1994). Therefore a sudden occlusion of a major branch of the coronary artery results in a rapid shift to anaerobic glycolysis, accompanied by an increase in myocardial glucose uptake and glycogen breakdown (Stanley *et al.*, 1997). A reduction in ATP inhibits the Na^+/K^+ -ATPase, resulting in increased intracellular concentrations of sodium and chloride ions, which causes cell swelling. Transport systems in the sarcolemma and sarcoplasmic reticulum become disrupted, producing an increase in cytosolic calcium. The resulting increase in calcium causes activation of proteases and alterations in contractile proteins. In the mitochondria pyruvate is not readily oxidised and lactate is produced, resulting in a

fall in intracellular pH, a reduction in contractile function, and a greater ATP requirement to maintain calcium homeostasis (Stanley, 2001).

1.2.3 Pathophysiology of AMI

Irreversible cell injury in AMI occurs after 20 minutes of total, no-flow ischaemia as a result of tissue necrosis. Ultra-structural changes occur within 10 minutes of the onset of ischaemia (Jennings *et al.*, 1974). The injury may be reversible with prompt reperfusion as long as the sarcolemma remains intact (Herdson *et al.*, 1965). After 20 minutes of ischaemia there is severe disruption of the sarcolemma as a result of osmotic swelling and phospholipase activation, causing a loss of the barrier function. Prolonged periods of ischaemia result in myocardial necrosis. The extent and rate of necrosis are determined by the duration and severity of ischaemia, the size of the myocardial bed-at-risk, and the amount of collateral blood flow available shortly after coronary occlusion (Fozzard *et al.*, 1991) (summarised in figure 1.1).

Irreversibly injured myocytes contain shrunken nuclei with marked chromatin margination. Irreversible ischaemic injury is characterised by the disruption and cell death of the sarcoplasmic reticulum. An increase in the cytosolic concentrations of calcium and mitochondrial impairment cause phospholipase activation and the release of phospholipids and free fatty acids, which are incorporated within the cell and damaged from reactive oxygen species. The cleavage of anchoring of cytoskeletal proteins and increases in cell membrane permeability, cause physical disruption and cell death (Jennings *et al.*, 1974).

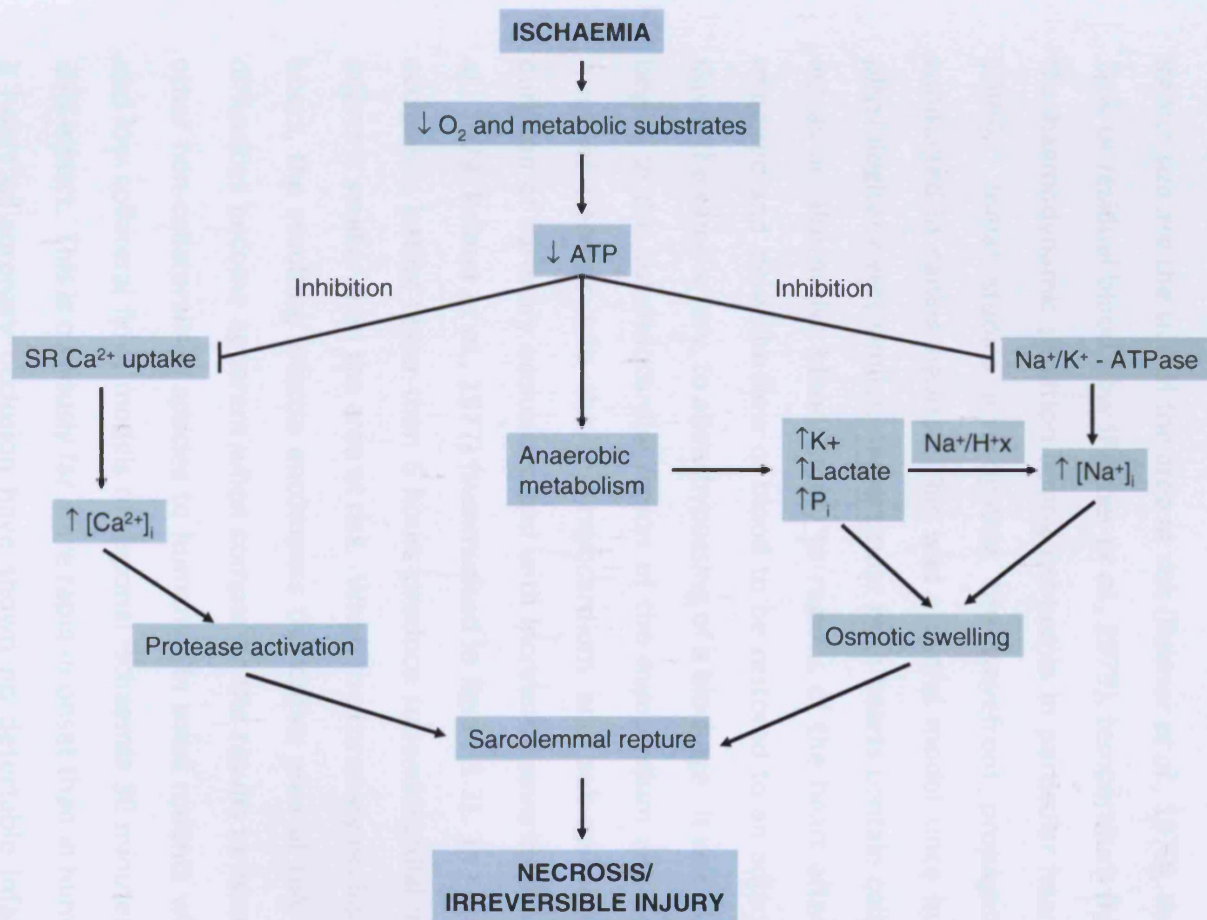


Figure 1.1 A summary of the biochemical and ionic changes that occur during myocardial ischaemia. If ischaemia occurs for a sufficient duration, it results in alterations in metabolism and ion fluxes, resulting in irreversible cell injury. Adapted from Ferdinandy et al. (2007).

1.2.4 Wavefront propagation of injury:

As with all experimental studies of human diseases, animal models are initially used to investigate the condition further. Typically rats, rabbits and dogs are used to model AMI, since this is the most humanely and economically viable option. However, this can pose difficulties with translating the results from animals to humans, since severity and size of infarct can vary between species. The major determinants of final infarct size are the size of the area at risk (Reimer *et al.*, 1979), the extent of collateral flow or residual blood flow (Reimer *et al.*, 1979), temperature (Miki *et al.*, 1998), and the haemodynamic situation during ischaemia in particular heart rate (Schulz *et al.*, 1995). Initial studies investigating the wavefront propagation of injury were conducted in canine hearts. This was a useful model since humans and dogs are physiologically very similar. In particular both hearts contain collateral vessels, which act as an alternative blood supply to regions of the heart after the main source is impaired and allow the flow of blood to be restored to an adjacent artery or further down the same artery, to allow bypassing of a blockage. It was observed that necrosis begins in the subendocardial region of the myocardium and progresses as a wave front of necrosis into the mid-myocardium and sub-epicardium with increasing duration of coronary occlusions and with increasing severity of ischaemia (Reimer *et al.*, 1979; Reimer *et al.*, 1977) (summarised in figure 1.2). In humans coronary artery occlusions lasting fewer than 6 hours produce subendocardial infarcts, in which the infarct is smaller than the area at risk. When the coronary occlusion period exceeds 6 hours, the resulting infarcts encompass the entire area at risk. However, greater difficulties become apparent when comparing the results between small rodents and other non-collateralised species to humans. In small rodents with a high heart rate and low collateral flow, models of regional ischaemia 30 minutes typically produce a 35% infarct. This is obviously far more rapid in onset than in humans, and in patients, 3 hours of coronary occlusion have shown no detectable infarction (Dixon *et al.*, 2002). These findings suggest that the physiology of the two species differs, with a major contributing factor perhaps being the existence of collateral vessels in humans which serve as a protective mechanism (Downey *et al.*, 2006). However, despite the difference in the duration of onset of ischaemic damage, animal models are still widely employed for investigating AMI, as the underlying pathophysiology is the same.

Therefore this allows data from animal models to be used with confidence and the results translated into humans. The other advantage of a more rapid onset of damage in small rodents such as rats, is that experiments can be conducted over shorter time periods and data obtained more quickly.

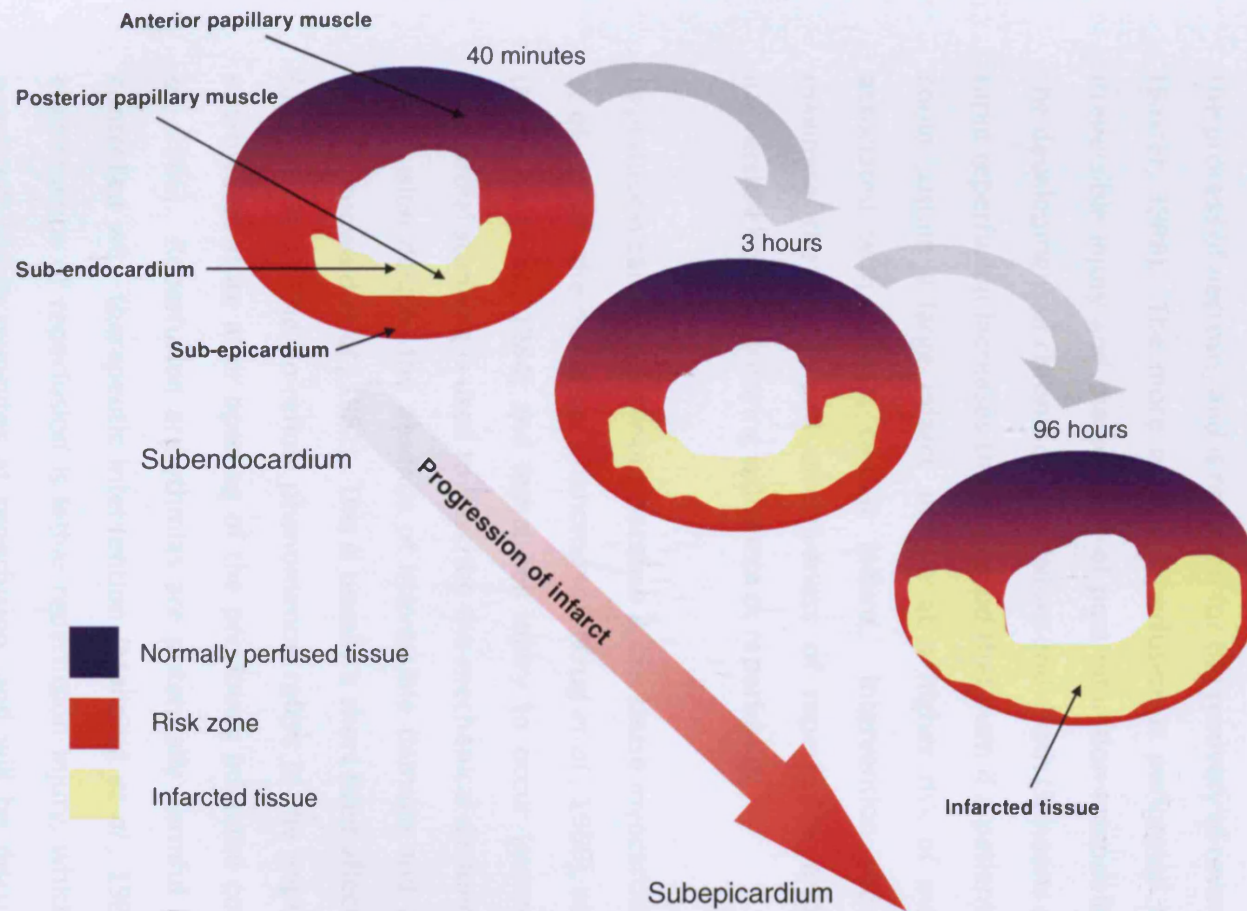


Figure 1.2 Transverse sections through infarcted heart tissue demonstrating the Wavefront phenomenon of cell death as described by Reimer and Jennings (Reimer et al., 1977). The infarct initiates in the subendocardium, due to the least collateral flow, and progresses across to the epicardium.

1.3 Reperfusion

Reperfusion is restoration of blood flow to the myocardium and is the only effective method of overcoming ischaemia. Reperfusion cannot occur in the absence of ischaemia. Paradoxically reperfusion is associated with both beneficial and deleterious components. Reperfusion is beneficial because it restores the oxygen and metabolic substrate supply, allows washout of accumulated metabolites, it may halt the process of necrosis, and is required for the recovery of reversibly injured myocytes (Baxter, 1998). The more quickly reperfusion is performed the less the extent of irreversible injury and the severity of post-infarction remodelling, which also effects the development of chronic cardiac failure and death (Simoons *et al.*, 1997). Failure of rapid reperfusion increases the likelihood that even if a patient survives an AMI, they could sustain a large infarct and be at a higher risk of morbidity and mortality associated with chronic cardiac failure. Intervention should therefore aim to maximise the speed and effectiveness of reperfusion and reduce the rate of irreversible cell injury during ischaemia or reperfusion.

Reperfusion can be deleterious because it can cause myocardial stunning (Braunwald *et al.*, 1982), the no-reflow phenomenon (Krug *et al.*, 1966), reperfusion arrhythmias (Manning *et al.*, 1984) and lethal cell injury to occur (summarised in figure 1.3). Myocardial stunning is used to describe the mechanical dysfunction of the heart after reperfusion despite the absence of irreversible damage and a restoration of blood flow (Braunwald *et al.*, 1982). This is usually a short lived effect from which the heart fully recovers. The no-reflow phenomenon refers to the impeded flow through the micro-vasculature after opening of the previously infarcted coronary artery (Krug *et al.*, 1966). Reperfusion arrhythmias are potentially harmful but can be effectively controlled with therapeutic intervention (Manning *et al.*, 1984). The most severe consequence of reperfusion is lethal reperfusion injury, which causes the death of previously viable myocytes at reperfusion, and will be discussed in greater detail below.

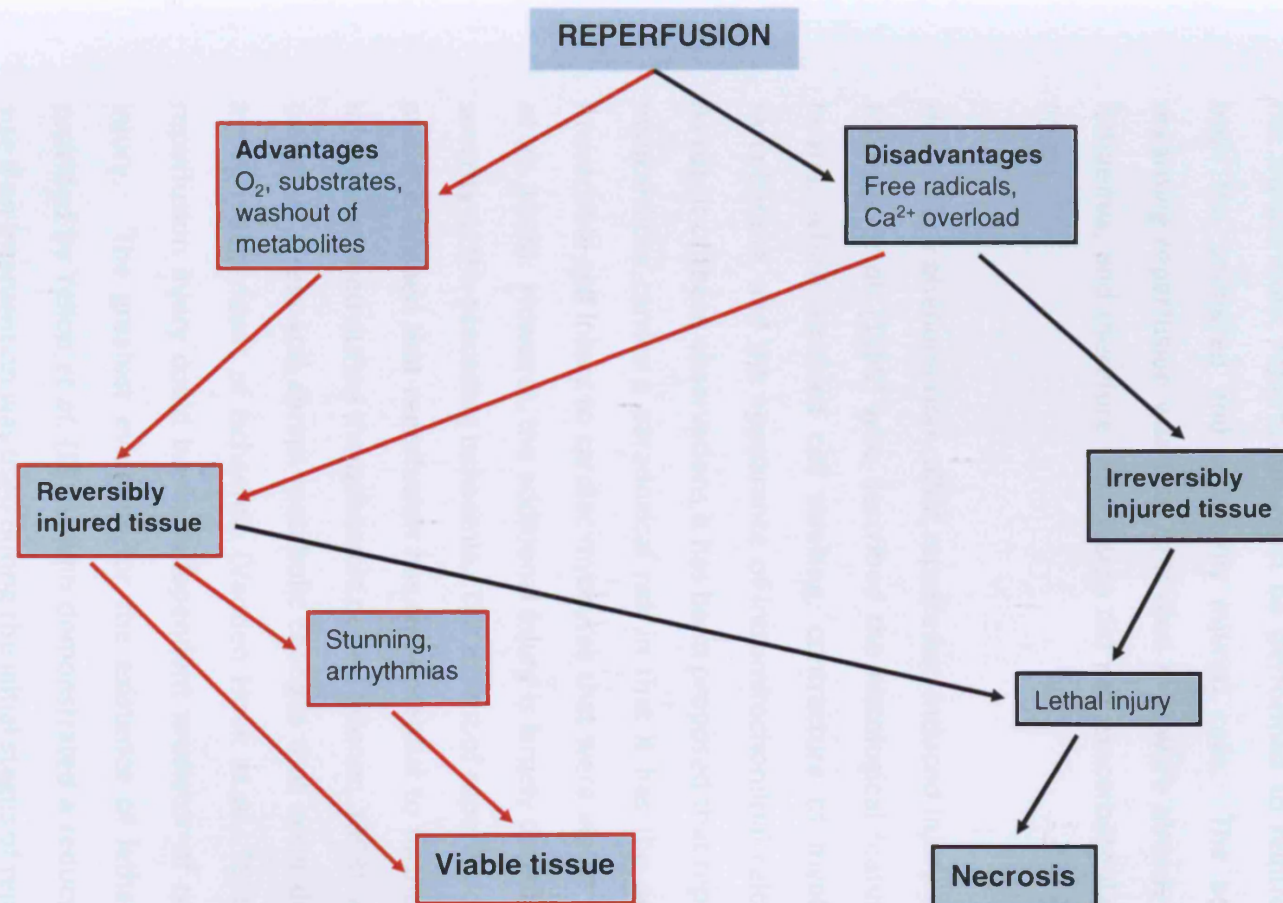


Figure 1.3 The balance of beneficial and deleterious components of reperfusion. The beneficial effects include restoration of oxygen and metabolic substrate supply and washout of accumulated proteins. The deleterious effects include generation of oxygen free radicals, calcium overload and damage of previously reversibly injured tissue. Adapted from Baxter (1998).

1.3.1 Lethal reperfusion injury

Initially it was viewed that cell death occurred during ischaemia due to the lack of ATP, which could not be produced by oxidative phosphorylation due to the anaerobic ischaemic environment (Ferdinandy *et al.*, 2007). Therefore in an attempt to salvage the myocardium, reperfusion must be performed to reintroduce oxygen to protect both the uninjured and reversibly injured cells. The original hypothesis posed regarding reperfusion was that cells died that were already irreversibly damaged by ischaemia, and therefore reperfusion did not exacerbate damage (Ferdinandy *et al.*, 2007).

However, a phenomenon called reperfusion-induced injury was proposed in 1960 by Jennings *et al.* (1960) who described the histological features of reperfused canine hearts, which included cell swelling, contracture of myofibrils, disruption of the sarcolemma, and the appearance of intramitochondrial calcium phosphate particles. As result of these observations it has been proposed that reperfusion of the ischaemic myocardium carries a paradoxical risk in that it has the potential to cause further irreversible cell injury to cardiac myocytes that were viable before reperfusion (Piper *et al.*, 1998). However, the additional injury is largely dependent on the duration and severity of the preceding ischaemia. The extent of reperfusion injury is highly debated and it is argued that reperfusion has the potential to further exacerbate the cellular injury sustained during the ischaemic period (Kloner, 1993), whilst others suggest that oxidative stress and abrupt metabolic changes that occur during reperfusion initiate injury independent of ischaemia (Vanden Hoek *et al.*, 1996), suggesting that lethal reperfusion injury could be an independent mediator of cell death from ischaemic injury. The greatest evidence for the existence of lethal reperfusion injury was provided by Yellon *et al.* (1999) who demonstrated a reduction in myocardial infarct size if an intervention was used during the initial stages of reperfusion.

The greatest difficulty with trying to measure lethal reperfusion injury is that it is not possible to accurately assess in situ the progress of necrosis during the transition from myocardial ischaemia to reperfusion (Piper *et al.*, 1998). Lethal reperfusion injury occurs as a result of the rapid restoration of oxygen, as well as factors such as

endothelial damage, platelet aggregation and neutrophil adhesion and activation. It can also be caused by massive calcium overloading of the metabolically impaired myocytes with damaged sarcolemma (Maxwell *et al.*, 1997) and the rapid restoration of physiological pH. If reperfusion occurs within 2 to 3 hours of the onset of ischaemia, the amount of salvage greatly exceeds the amount of irreversible reperfusion injury (Buja, 2005). The additional damage to the myocardium caused by lethal reperfusion injury often detracts from the potential benefit of reperfusion compared to an un-reperfused heart.

There is also increasing evidence to suggest that myocardial infarct size can increase in relation to the duration of reperfusion. This suggests the possibility of a wavefront of myocardial reperfusion injury caused by apoptosis and inflammation (Zhao *et al.*, 2000a), which could be reduced by a late intervention of antiapoptotic and anti-inflammatory agents (Doukas *et al.*, 2006).

1.3.2 Proposed mediators of lethal reperfusion injury

Several potential mediators of lethal reperfusion injury have been proposed and will be discussed briefly. The oxygen paradox suggests that the generation of reactive oxygen species (ROS) at reperfusion produces damage to the myocardium to a greater extent than ischaemia alone (Hearse *et al.*, 1973). However, there is the lack of consistent experimental evidence to support this theory. The calcium paradox suggests that sarcolemmal-membrane damage and oxidative stress induced damage to the sarcoplasmic reticulum, results in an abrupt increase in calcium due to the inability to regulate the intracellular concentration. Intracellular and mitochondrial calcium overload occur, resulting in hypercontracture of cardiac cells (Bolli *et al.*, 2004). Again, inconclusive experimental evidence surrounds this theory. The pH paradox proposes that the rapid restoration of physiological pH contributes to lethal reperfusion injury (Lemasters *et al.*, 1996), but clinical studies delaying the restoration of physiological pH have been ineffective (Avkiran *et al.*, 2002). The accumulation of inflammatory mediators at reperfusion into the infarct zone and subsequent migration into the myocardial tissue, with the release of degradative enzymes and ROS is another proposed mechanism of lethal reperfusion injury (Vinten-Johansen,

2004). Clinical trials have shown some promise with an 11% reduction in infarct size following use of an anti-inflammatory agent, adenosine, at reperfusion (Ross *et al.*, 2005). However, in addition to these paradoxes, the mitochondrial permeability transition pore (mPTP) has been proposed as a key mediator of reperfusion injury, for the reasons discussed below. However, mPTP is probably not exclusively the causative factor of lethal reperfusion, and intra-cellular calcium over-load and ROS generation (Opie, 1989) are probably contributing factors too.

1.3.3 Role of the mitochondrial permeability transition pore in reperfusion injury

Greater understanding of the role of the mitochondria in determining cell fate during and after cellular stresses, has led to the discovery that reperfusion favours opening of the mPTP, a pore on the mitochondrial inner and outer membrane which when open results in cell death due to uncoupling of oxidative phosphorylation and loss of membrane potential (Halestrap *et al.*, 2003). The idea that the mPTP may play a role in cardiac reperfusion injury is not a new concept and was first proposed by Crompton *et al.* in 1987 (Crompton *et al.*, 1987).

Opening of the mPTP at reperfusion occurs due to the favourable environmental conditions. The rapid restoration of oxygen at reperfusion causes re-activation of the mitochondria and electron transport chain, resulting in a significant production of ROS and further ROS in a ROS-induced-ROS release (Griffiths *et al.*, 1995). Reactive nitrogen species (RNS), for example peroxynitrite (ONOO^-), are also generated from the presence of nitric oxide. These reactive species causes oxidative damage to cellular structures such as the sarcoplasmic reticulum, resulting in calcium release. ATP production is restored allowing activation of the sodium/calcium ($\text{Na}^+/\text{Ca}^{2+}$) exchanger, resulting in calcium overload. The combined effect of calcium accumulating in the mitochondrial matrix, along with ROS and RNS, and the increase in pH due to hydrogen ions being washed out, provides favourable conditions for the formation and opening of the mitochondrial permeability transition pore. Allowing the mPTP to open during the first few moments of reperfusion, results in cell death via necrosis due to the uncoupling of oxidative phosphorylation and collapse of the

mitochondrial membrane potential, resulting in mitochondrial swelling and a sustained lack of ATP production (Hausenloy *et al.*, 2003).

Crompton *et al.* (1988) demonstrated that inhibition of opening of the mPTP with cyclosporine A induced cardioprotection. Further investigation by Halestrap's laboratory (Griffiths *et al.*, 1995) showed that the mPTP remained closed during ischaemia and only opened during the initial stages of reperfusion. Infarct size limitation with mPTP inhibitors at reperfusion provided further evidence for inhibiting opening of the pore to produce cardioprotection (Hausenloy *et al.*, 2003). If the pore is kept in a closed state it protects the myocardium against reperfusion injury (Hausenloy *et al.*, 2002). However, it has been shown that inhibiting opening of the pore is ineffective if it occurs after the initial few minutes of reperfusion, suggesting a critical time period for inducing cardioprotection (Hausenloy *et al.*, 2003). There appears to be substantial evidence to support the role of mPTP opening at reperfusion induces ischaemia-reperfusion injury (Halestrap *et al.*, 2000b) and that to enable complete recovery from ischaemia-reperfusion the mitochondria must return to full functioning and the mPTP be kept in closed state.

Following on from studies in animals that demonstrated a 50% reduction in infarct size when inhibiting mPTP opening at reperfusion (Hausenloy *et al.*, 2003), has led to proof-of-concept clinical trials to be undertaken. These trials discussed in more detail below (section 1.7.2), will hopefully confirm the results shown in animal models, and assist with clarification of the mediator role of the mPTP in lethal reperfusion injury.

1.3.4 Composition of the mPTP

The exact composition of the mPTP still remains unclear, although several components have been identified which appear essential for its function. These include the voltage dependent anion channel (VDAC), adenine nucleotide translocator (ANT) and cyclophilin-D (CYP-D) (Halestrap *et al.*, 2004). CYP-D knock-out mice showed an increased resistance to mPTP opening and infarct size limitation compared to wild type mice (Baines *et al.*, 2005; Nakagawa *et al.*, 2005). However, the clinical use of cyclosporine A as a cardioprotective agent is limited by concerns about its

dosing and side effects (Hausenloy *et al.*, 2008). This may limit the drug to a single intravenous bolus that could be administered to patients undergoing myocardial reperfusion after an AMI or in patients receiving coronary artery bypass angioplasty surgery, or angioplasty.

1.4 Modes of cell death

Originally it was proposed that there were four main types of cell death, oncosis, necrosis, apoptosis, and autophagy involved in both the maintenance of normal cardiac homeostasis and in the development of cardiac and vascular diseases (Buja, 2005). It appears that multiple modes of cell death can participate simultaneously in various pathological processes (Kostin, 2005). Environmental factors play a large part in determining which process of cell death occurs, in particular the availability of ATP to drive active processes such as apoptosis. However, since Majno *et al.* (1995) reviewed the modes of cell death, the Society for Toxicologic Pathologists recommended that the term necrosis should only be used to describe the changes that occur after cell death, regardless of the pathway by which the cell originally died (Levin *et al.*, 1999). The modifiers “apoptotic” or “oncotic” should be used to specify the predominant cell death pathway.

1.4.1 Oncosis

Oncosis is non-programmed form of cell death characterised by swelling, disruption of the sarcolemma and the mitochondria, chromatin clumping, blebbing and removal of cells by inflammatory mediators (Majno *et al.*, 1995). It is a passive response to external noxious stimuli (Majno *et al.*, 1995) such as ischaemia-reperfusion and hypoxia.

1.4.2 Apoptosis

Apoptosis is an ATP dependent conserved form of self-destruction, resulting in cell and nuclear shrinkage and fragmentation without external inflammation (Kerr *et al.*, 1972). It is characterised by preservation of mitochondrial and sarcolemmal integrity, nuclear chromatin condensation, removal of debris by macrophages or neighbouring

cells (Majno *et al.*, 1995), nuclear fragmentation, blebbing, and phosphatidylserine exposure on the cell surface of the plasma membrane (Zamzami *et al.*, 1996). Apoptosis can be stimulated by both a receptor mediated (extrinsic) and a mitochondrial (intrinsic) pathway in acute and chronic cardiac disease (Borutaite *et al.*, 2003a). Both pathways are operative in cardiomyocytes. One of the main characteristics of apoptosis is the cleavage of chromosomal DNA into nucleosomal units. The activation of a specific class of proteases called the caspases (cytosolic aspartate residue-specific cysteine proteases) are required for this process because they activate DNases, inhibit DNA repair enzymes, and cause the break down of structural proteins in the nucleus (Kroemer *et al.*, 2007). Most cell death in vertebrates occurs via the mitochondrial pathway (Green *et al.*, 2004). Formation of the mPTP causes the release of cytochrome c, which is a key mediator of caspase activation, highlighting the critical role the formation of the mPTP plays in apoptosis (Scaffidi *et al.*, 1998).

1.4.3 Necrosis

Necrosis is a degradative process that follows the onset of irreversible injury via oncosis and apoptotic forms of cell death (Majno *et al.*, 1995). Cells die from necrosis when they cannot maintain adequate ATP levels, in contrast to apoptosis which requires ATP to activate the caspase cascade (Weiss *et al.*, 2003). Necrotic cells gain volume which leads to rupture of the plasma membrane and the unorganised dismantling of swollen organelles (Kroemer *et al.*, 2007). Necrosis lacks a specific biochemical marker and can only be detected by electron microscopy. It is also considered harmful because it is also often associated with local inflammation, which can lead to tumour growth (Vakkila *et al.*, 2004).

1.4.4 Modes of cell death during ischaemia-reperfusion

The primary pathological expression of coronary artery disease is myocardial cell injury resulting from AMI (Skyschally *et al.*, 2008). Injury can range from a small insult with no permanent damage, to a large insult resulting in alteration of cardiac function and ultimately cell death (Skyschally *et al.*, 2008). The duration and severity of

ischaemia appears to be the key factor in the resulting insult. An ischaemic insult causing cardiac myocytes to become irreversibly injured will result in cell death. The modes of cell death elicited during ischaemia-reperfusion will now be addressed.

Ischaemia causes progressive ultrastructural and functional damage to the mitochondria as the duration and intensity increases (Lesnefsky *et al.*, 1997). Since cardiac myocytes are highly aerobic cells, the mitochondria are vital to cell survival as they are the primary source of ATP production via oxidative phosphorylation (Solaini *et al.*, 2005). Since the ischaemic environment is highly anaerobic (Dobson *et al.*, 2002), oxidative phosphorylation cannot occur and there is the rapid depletion of ATP during the initial stages of ischaemia (Javadov *et al.*, 2007). Initially glycolysis can be used to maintain ATP levels, but this is short lived due to a rise in H^+ which prevents glycolytic enzyme activity (Solaini *et al.*, 2005) and also causes the mPTP to remain closed (Javadov *et al.*, 2007). This suggests that oncosis and subsequent necrosis would be the favoured route of cell death during ischaemia, since they are passive processes.

Evidence investigating the role of oncosis during ischaemia in the absence of reperfusion is limited, but several studies suggest a role. Takemura *et al.* (1997) showed that apoptosis was absent in rabbit hearts after coronary occlusion, through the use of immunogold dUTP nick-end labelling (TUNEL) staining and electron microscopy, whilst a study by Ohno *et al.* (1998) suggests that apoptosis does not play a role in myocardial infarction. Ischaemia induced in human myocardial cells also demonstrated fragmentation indicative of the occurrence of oncosis (Itoh *et al.*, 1995).

However, despite oncosis/necrosis being a logical route of cell death, there is much experimental to suggest a role of apoptosis during ischaemia. Borutaite *et al.* (2003b) used TUNEL positive staining to demonstrate that apoptosis occurred during 60 minutes of global ischaemia in the absence of reperfusion. They did however only show that 2% of myocytes underwent apoptosis, and went on to demonstrate that a far greater number of apoptotic cells were present during reperfusion. These findings

suggest that apoptosis may be initiated during ischaemia, but cannot actually occur to completion until reperfusion when ATP levels are restored. There are in fact very few studies that support a role of apoptosis during ischaemia alone (Fliss *et al.*, 1996). Since apoptosis is an ATP dependent process it may explain why it is more likely to occur at reperfusion when there is a restoration of ATP production (Leist *et al.*, 1997). In studies investigating ischaemia following by reperfusion, there is a greater incidence of apoptosis reported, suggesting that reperfusion accelerates the apoptotic death process initiated during ischaemia (Fliss *et al.*, 1996; Freude *et al.*, 2000; Gottlieb *et al.*, 1994; Scarabelli *et al.*, 1999).

Opening of the mPTP at reperfusion has been implicated to produce a switch from reversible to irreversible cell injury (Halestrap *et al.*, 2004). Mitochondrial dysfunction has been shown to affect cell viability in several ways. It causes a loss of ATP synthesis, increases ATP hydrolysis, impairs ionic homeostasis, causes the production of ROS, and the release of pro-apoptotic proteins (Kroemer *et al.*, 1998). These are all factors involved in irreversible damage, suggesting that the mitochondria are involved in apoptosis and necrosis. There appears to be growing evidence to suggest that the level of mitochondrial damage and duration of opening of the mPTP at reperfusion determines the fate of cardiac myocytes (Crompton, 1999). Brief opening of a limited number of pores will produce far less damage than a generalised prolonged opening, which will result in cessation of ATP production and cell death via necrosis. An intermediary stage may exist whereby transient and localised opening of mPTP allows apoptotic proteins, such as cytochrome C, to be released initiating the apoptotic cascade (Halestrap *et al.*, 2000a). There is also strong evidence to suggest that the necrotic centre of an infarct is surrounded by a peripheral ring of apoptosis (Takemura *et al.*, 2004) perhaps where mitochondrial damage was less severe and apoptosis could occur. Early studies by Crompton *et al.* (1987; 1988) suggest a role of necrosis during ischaemia-reperfusion mediated by opening of the mPTP.

Apoptosis has also been reported to only occur during reperfusion, or to become the dominant form of cell death after reperfusion (Gottlieb *et al.*, 1994; Zhao *et al.*, 2000b). Zhao *et al.* (2001b) demonstrated that necrotic cell death peaked after 24

hours of reperfusion and apoptotic cell death increased up to 72 hours of reperfusion, presumably once the oxidative phosphorylation was fully restored. Other studies have shown that inhibition of the apoptotic signalling cascade during reperfusion is able to attenuate the apoptotic and necrotic components of cell death, suggesting that apoptosis can evolve into necrotic cell death (Zhao *et al.*, 2003c). Inhibition of apoptosis at reperfusion has also been shown to improve contractile function of ischaemic canine hearts (Zhao *et al.*, 2003c).

An interesting study by McCully *et al.* (2004) compared the relative contribution of apoptosis and necrosis to myocardial ischaemia-reperfusion injury. They reported through the use of TUNEL positive staining, tetrazolium staining, and caspase activity, that during global ischaemia lasting up to 30 minutes in isolated rabbit hearts, necrosis contributed significantly more to infarct than apoptosis. Apoptosis was also shown to be significantly decreased by inhibition of caspases, which are an essential component of apoptosis, during early reperfusion. However, surprisingly this did not lead to an improvement in the immediate post-ischaemic functional recovery.

Scarabelli *et al.* (1999) were the first to quantify apoptosis in cardiac myocytes using TUNEL positive and counter staining. They showed that apoptosis predominantly affects endothelial cells during early reperfusion and that these cells undergo apoptosis before cardiac myocytes. These findings are highly important because they suggest that soluble factors may be released from the injured vascular cells which diffuse into the myocardium during reperfusion, contributing to cell death. This suggests that apoptosis could occur in cardiac myocytes during reperfusion despite opening of the mPTP. Knock-out mouse models lacking various apoptotic components have shown between a 50 and 65% reduction in infarct size, providing further evidence for a role of apoptosis in cell death in ischaemia-reperfusion (Garg *et al.*, 2003; Takashi *et al.*, 2000).

Whilst reviewing the literature about the role of apoptosis in ischaemia-reperfusion injury, it appears that there is much evidence to support its role. However, a large number of studies measure apoptotic mediators as opposed to using staining

techniques to observe the hallmark effects of apoptosis. This implies that whilst apoptotic mediators may be released, whether they actually initiate apoptosis is questionable. Interestingly, apoptotic cell death can be shifted to a more necrotic phenotype by the removal of essential caspase activators such as APAF-1 (Golstein *et al.*, 2005). This suggests that during ischaemia-reperfusion whilst the release of some apoptotic mediators may occur, other key components may not be released shifting apoptosis towards a necrotic death pathway. With this in mind it appears that oncosis/necrosis is a far more likely form of cell death during ischaemia and the early stages of reperfusion, than apoptosis. Whilst apoptosis initiated during the early stages of ischaemia or even reperfusion, may contribute to cell death during the later stages of reperfusion after the restoration of oxidative phosphorylation, providing ATP to drive the process. However, what remains clear is that there is still great uncertainty surrounding the relative contributions of apoptosis and necrosis to cell death, and whether the two processes are related.

1.5 Cardioprotection

Cardioprotection refers to the prevention of stress-induced myocardial injury. This term has been used to describe the protection against ischaemia-reperfusion induced myocardial injury. Cardioprotection at reperfusion is an important clinical target because the onset of AMI cannot be predicted. This means that therapeutic intervention can only occur once AMI is initiated during the final moments of ischaemia and initial stages of reperfusion. Since both ischaemia and reperfusion have been reported to cause injury to the heart, with reperfusion possibly extenuating that caused during ischaemia, limiting the overall damage to the myocardium is of great importance to preserve its structural integrity and maintain maximum function.

1.5.1 Historical background to the reperfusion injury salvage kinase (RISK) pathway

1.5.1.1 Anti-apoptotic effects of PI3K and Akt

It has long been recognised that activation of the protein and lipid kinase cascade phosphoinositide 3-kinase (PI3K) and its downstream effector serine-threonine kinase (Akt), produces a potent stimulus for cell proliferation, growth and survival in many systems of the body (Matsui *et al.*, 2003). Activation of PI3K subsequently causes phosphorylation of membrane phosphatidylinositol 4,5-bisphosphate, producing phosphatidylinositol 3,4,5-triphosphate (PIP3), some of which is converted to phosphatidylinositol 3,4-bisphosphate (PIP2) by an inositol phosphate (Damen *et al.*, 1996). PIP2 and PIP3 accumulate in the cell membrane and recruit Akt and PDK1 to the cell membrane, resulting in phosphorylation and activation of Akt (Vanhaesebroeck *et al.*, 2000). Perhaps the most interesting feature of PI3K and Akt activation in the context of cardiomyocytes is the antiapoptotic effect. Insulin-like growth factor (IGF1) has been shown to activate PI3K (Kulik *et al.*, 1997) and Akt, and activation of PI3K is essential for the anti-apoptotic effects of IGF1 (Matsui *et al.*, 1999). Most importantly it has been demonstrated that acute activation of PI3K or Akt is sufficient to inhibit cardiomyocyte apoptosis (Matsui *et al.*, 1999). Adenoviral gene transfer of constitutively active Akt to the heart has been shown to cause a significant reduction in cardiomyocyte apoptosis and infarct size *in vivo* (Matsui *et al.*, 2001). Interestingly inhibition of Akt activity with a dominant negative construct has been shown to accelerate hypoxia-induced dysfunction of cardiomyocytes (Matsui *et al.*, 2001). These findings provide evidence that acute activation of Akt provides both cardioprotection and improved function of cardiomyocytes (Matsui *et al.*, 2003). PI3K is thought to play a critical role in organ size and development (Leesters *et al.*, 1996) and transgenic cardiac over-expression of either the constitutively active or dominant negative forms has demonstrated its role in the modulation of cardiomyocyte growth (Shioi *et al.*, 2000). Transgenic over-expression of activated Akt in the heart has been shown to induce hypertrophy (Matsui *et al.*, 2002) suggesting it may mediate the effects of cardiac growth. Taken together these findings demonstrate that PI3K and Akt are important pro-survival kinases in the myocardium and acute activation produces cardioprotection due to their anti-apoptotic function.

Extending the pro-survival effects of PI3K and Akt in the myocardium, in particular the finding that constitutively expressed PI3K and Akt limits hypoxia-reoxygenation induced apoptosis, led to the proposal of the reperfusion injury salvage kinase (RISK) pathway in the late 1990's. This concept stems from the hypothesis that apoptotic cell death was contributing to lethal reperfusion injury, and the existence of the pro-survival anti-apoptotic protein kinases Akt and Erk1/2, which when specifically activated at the time of reperfusion produced cardioprotection (Yellon *et al.*, 1999). Many subsequent studies have supported this hypothesis and have shown the involvement of anti-apoptotic effectors downstream of Akt including phosphorylation of proapoptotic proteins, such as BAX and BAD, the inhibition of caspase 3 activation, and the phosphorylation and activation of p70s6K. p70s6K acts to inhibit BAD and also activates Bcl-2 a proapoptotic protein (Harada *et al.*, 2001), although as previously explained, the quantitative contribution of apoptosis to the overall cell death in AMI may be limited.

There is now a continually expanding list of pharmacological agents that have been shown to activate the RISK pathway to induce cardioprotection. Further investigation into the RISK pathway has revealed that it can be primed for activation prior to reperfusion (preconditioning) and also at reperfusion (postconditioning). Also the identification of cardioprotective kinases involved in the RISK pathway is continually expanding and now includes protein kinase C (PKC), protein kinase G (PKG), phosphoprotein 70 kDa ribosomal protein S6 kinase (p70s6K), and Glycogen synthase kinase 3 beta (GSK-3 β).

1.5.1.2 Role of inhibition of the mitochondrial permeability transition pore (mPTP) in cardioprotection

The original hypothesis that activation of the RISK pathway produced cardioprotection by the recruitment of anti-apoptotic pathways has been extended further since the discovery that activation of the RISK pathway also causes cardioprotection by the inhibition of the mitochondrial permeability transition pore (mPTP) opening (Juhaszova *et al.*, 2004). As already mentioned, the mPTP is a non-selective conductance pore of the mitochondrial inner membrane (Griffiths *et al.*, 1995).

Opening of the mPTP channel at reperfusion has been shown to mediate cell death in the first few minutes of reperfusion (Hausenloy *et al.*, 2002). However, if the pore is kept in a closed state this induces cardioprotection. Once it had been established that there was a link between activating the RISK pathway and inhibition of the mPTP to induce cardioprotection, exactly how these two components were linked was investigated.

Prevention of mPTP opening occurs as a downstream event of the RISK pathway components discussed previously. Specifically activation of PKG via cGMP, which in turn activates PKC- ϵ , causes opening of the ATP-dependent mitochondrial permeability potassium channel (K_{ATP}) (Costa *et al.*, 2005). Opening of the channel results in the production of reactive oxygen species (ROS) (Andrukhiv *et al.*, 2006) which perform two functions. The first is to activate protein kinases in the cytosol such as Akt, Erk1/2, p38 mitogen activated protein kinases, and PKC. This relays the cardioprotective signal to protect the myocardium after a prolonged ischaemic episode (Yellon *et al.*, 2003). The second role of the ROS is to inhibit the opening of the mitochondrial permeability transition pore (mPTP) (Costa *et al.*, 2006) at reperfusion. Therefore activation of the RISK pathway specifically during the first few minutes of reperfusion is believed to be cardioprotective by attenuating reperfusion induced cell death via anti-apoptotic mechanisms. This suggests the pro-survival kinase cascades would be a suitable target to manipulate and upregulate during early reperfusion in order to limit reperfusion induced cell death. A summary of the kinases and proposed mechanism involving inhibition of mPTP opening at reperfusion is shown in figure 1.4.

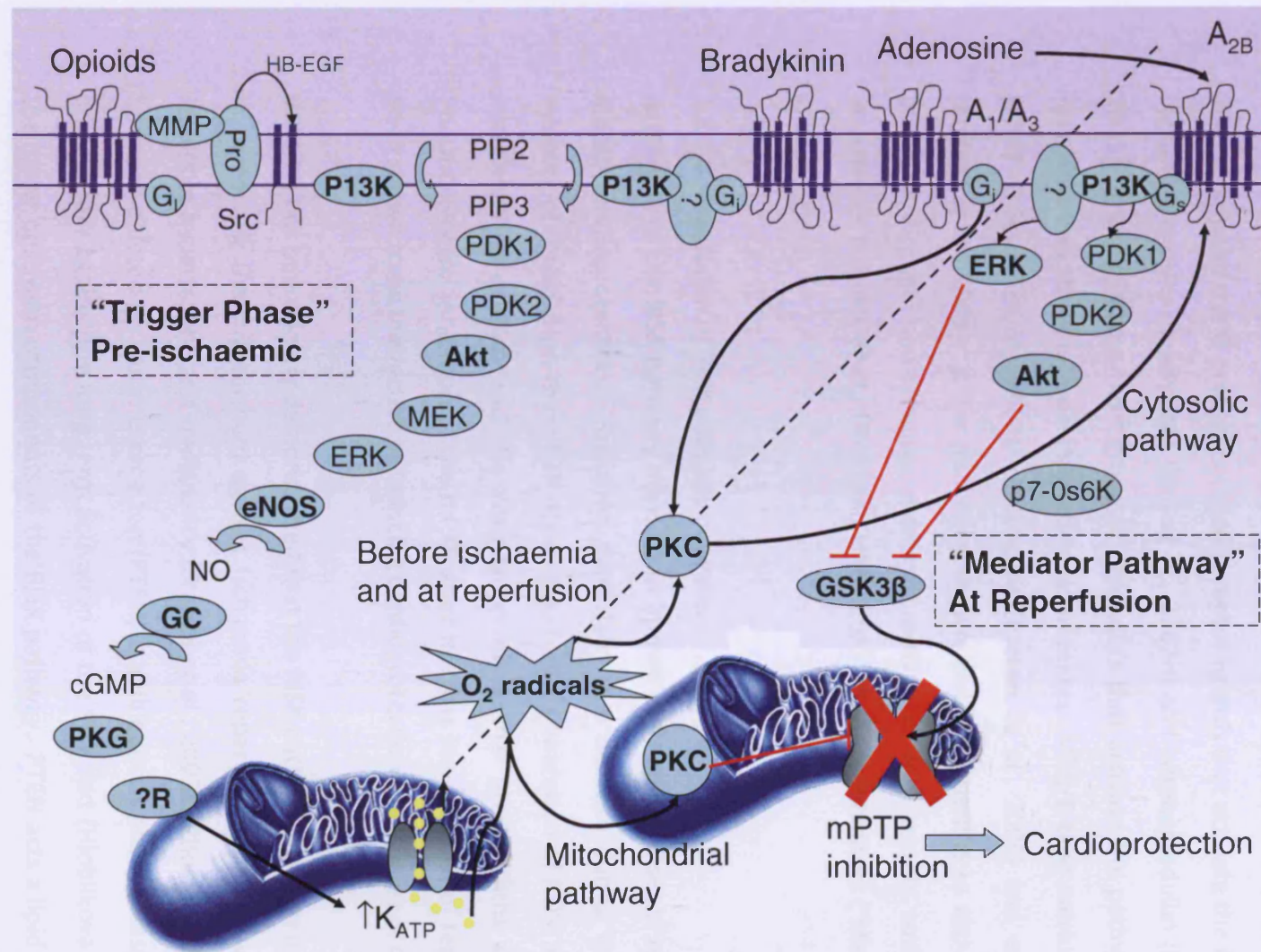


Figure 1.4 The RISK pathway consists of the pre-ischæmic "Trigger phase" which primes the pathway prior to ischaemia. At reperfusion the "Mediator pathway" is activated, comprised of pro-survival kinases which prevent opening of the mPTP and induces cardioprotection. Adapted from Costa et al. (2008)

1.5.1.3 Activators of the RISK pathway

The RISK pathway is activated in response to a wide range of receptors, including growth factors and G-protein coupled receptor ligands. Examples of growth factors are transforming growth factor- β 1, insulin and corticotrophin-1 (Hausenloy *et al.*, 2007). Some of the G-protein coupled receptor ligands that activate the RISK pathway include adenosine, bradykinin (Bell *et al.*, 2003b) and adrenomedullin (Hamid *et al.*, 2005). There is also a third group of mediators that activate the pathway via other receptors and these include the natriuretic peptides (ANP)/B-type natriuretic peptide (BNP) (D'Souza *et al.*, 2003), oestrogens (Patten *et al.*, 2004) and erythropoietin (Bullard *et al.*, 2005). It has also been shown that it is possible to activate the RISK pathway through non-receptor mediated activation with agents such as volatile anesthetics (Schlack *et al.*, 1998) and HMG-CoA reductase inhibitors ("statins") (Bell *et al.*, 2003a).

1.5.1.4 Regulation of RISK pathway activation

Activation of the RISK pathway must occur immediately at the onset of reperfusion to induce cardioprotection. This time point is critical because during the initial few minutes of reperfusion the mPTP opens due to the generation of ROS, an increase in mitochondrial calcium and the restoration of normal pH (Griffiths *et al.*, 1995). Pharmacological inhibition of the mPTP after the first few minutes of reperfusion has been shown to be ineffective at inducing cardioprotection (Hausenloy *et al.*, 2003)

Whilst it has been clearly demonstrated that the RISK pathway plays an important role in protecting the myocardium against ischaemia reperfusion injury, over-activation results in hypertrophy and malignancy (Franke *et al.*, 2003). Phosphatase and tensin homolog deleted on chromosome ten (PTEN) is a ubiquitous phosphatase that acts to protect cells against the long term activation of the PI3K/Akt (Hlobilkova *et al.*, 2003), the major upstream components of the RISK pathway. PTEN acts as a lipid phosphatase (Leslie *et al.*, 2004), causing the reversal of the phosphorylation of the second messenger phosphatidylinositol (4,5)-bisphosphate (PIP₂) into phosphatidylinositol (3,4,5)-trisphosphate (PIP₃), which would normally act downstream to activate 3-phosphoinositide-dependent kinase 1 (PDK-1) and subsequently Akt activation

(Mocanu *et al.*, 2007) (as shown in figure 1.4). Recently it has been shown that PTEN is down regulated after 15 minutes of ischaemia and 30 minutes of reperfusion in an isolated rat heart model (Cai *et al.*, 2005). Whilst this study used a relatively mild ischaemic episode, it does highlight the potential for acute down-regulation of PTEN, to increase RISK pathway activation and enhance endogenous cardioprotection.

1.5.1.5 Detrimental protein kinases

Although the RISK pathway consists of a cascade of pro-survival kinases, there are also deleterious components. For example Rho-kinase (ROCK) has been shown to be activated by ischaemia-reperfusion. Its mode of action is to regulate the RISK pathway and overactivation causes cardiovascular damage by inhibiting the RISK pathway (Noma *et al.*, 2006). Hamid *et al.* (2007) demonstrated that inhibition of ROCK, specifically at the time of reperfusion, produced cardioprotection through an Akt/eNOS-dependent mechanism. These findings suggest that ROCK activation at reperfusion may be deleterious through suppression of the RISK pathway.

1.6 Activating the RISK pathway to induce cardioprotection

The RISK pathway and subsequent cardioprotection can be activated by two cardioprotective algorithms: ischaemic preconditioning (IPC) and ischaemic postconditioning (IPOST). Both involve one or more short bursts of ischaemia-reperfusion either prior to a prolonged ischaemic episode, IPC, or at the beginning of reperfusion, IPOST, to induce a reduction in damage to the myocardium. More recently it has been shown that pre and post-conditioning utilise many of the same kinases of the RISK pathway, and that both mechanisms of cardioprotection cause inhibition of mPTP opening at reperfusion. This finding has provided increased potential cardioprotective targets, as the knowledge of the RISK pathway and its components are expanded. The concepts of IPC and IPOST will now be addressed and the potential cardioprotective mechanisms that cause activation of the RISK pathway will be discussed and explored.

1.6.1 Ischaemic preconditioning

The most interesting feature of the RISK pathway is that various components such as Akt and Erk1/2 p38 MAPK can be primed for activation prior to a prolonged ischaemic period, to induce cardioprotection at reperfusion. This is achieved by ischaemic preconditioning (IPC), a cardioprotective phenomenon produced by sub-lethal short periods of coronary occlusion and reperfusion prior to a prolonged ischaemic event. It was first described by Murry *et al.* (1986) who demonstrated that infarct size could be reduced in anaesthetised dogs subjected to four, 5 minute periods of left anterior descending coronary artery occlusion, with 5 minutes periods of reperfusion between, prior to 40 minutes of occlusion of the same artery. The cardioprotective effect observed was independent of changes in transmural myocardial blood flow and it was proposed that the effect was as a result of a rapid metabolic adaptation of the ischaemic myocardium. Ischaemic preconditioning has been shown to be reliably reproducible in a variety of species using varying protocols, resulting in its acceptance as the “gold standard” for cardioprotection. “Classical” preconditioning, as just described, is a short lived transient effect and is lost if the interval between preconditioning and prolonged ischaemic episode is greater than 60 minutes. A second window of protection exists between 24 and 72 hours of the preconditioning stimulus (Kuzuya *et al.*, 1993). This was designated the title “late phase preconditioning” to distinguish it from “classical” preconditioning.

1.6.1.1 Mechanism of action of ischaemic preconditioning

It is thought that during IPC there is the generation of endogenous autacoid ligands, such as adenosine (from breakdown of ATP), bradykinin, opioids, prostacyclin, NO, reactive oxygen species and TNF- α (Gross *et al.*, 2006). These endogenous ligands accumulate during the preconditioning stimulus and bind to their respective GPCRs to trigger the preconditioned state by activating PKC. Adenosine couples directly to PKC through the phospholipases. The EGF receptor (EGFR) couples muscarinic and opioid receptors to PI3K (Critz *et al.*, 2005). EGF receptor transactivation requires metalloproteinase (MMP) cleavage from its membrane bound precursor pro-heparin-binding EGF (HB-EGF) (Krieg *et al.*, 2004). This then gives rise to activation of a complex pathway that includes PI3K, Akt, NO synthase, guanylyl cyclase, PKG, opening

of mitochondrial K_{ATP} channels, activation of PKC by redox signalling, and ultimately results in inhibition of the mPTP opening at reperfusion (see figure 1.4) (Davidson *et al.*, 2006; Gross *et al.*, 2006; Hausenloy *et al.*, 2002; Hausenloy *et al.*, 2005a; Hausenloy *et al.*, 2004; Javadov *et al.*, 2003; Lecour *et al.*, 2005; Matsumoto-Ida *et al.*, 2006; Solenkova *et al.*, 2006). It is unknown if bradykinin signals through an EGF receptor or whether it activates a different metalloproteinase to activate PI3K and the subsequent downstream effectors (Critz *et al.*, 2005). The signalling events that occur during IPC are therefore considered of importance at reperfusion.

The exact mechanism of RISK pathway recruitment at reperfusion is unclear, but several mechanisms have been proposed. A possible mechanism of protection induced by IPC is that kinase activation, initiated by ischaemic preconditioning, needs to be sustained into reperfusion to induce cardioprotection. It has been shown that Akt activation needs to be continued for the initial 50-60 minutes of reperfusion (Solenkova *et al.*, 2006). However, whether activation of kinases can be maintained during ischaemia is questionable. Interestingly at reperfusion adenosine receptors must be repopulated for IPC to protect the heart, this is presumed to be through an A_{2B} receptor (Solenkova *et al.*, 2006). The threshold for activation of these low affinity receptors is lowered by PKC activation, allowing endogenous adenosine released during ischaemia to be protective during ischaemia. Recently Lim *et al.* (2007) proposed that during the ischaemic preconditioning stimulus there may be non-pathological opening of the mPTP, inducing a mild form of mitochondrial stress, which acts as a trigger for adaptation. Therefore the mPTP could be acting as a mediator of cardioprotection prior to ischaemia.

1.6.1.2 Clinical intervention with ischaemic-preconditioning

The potential of IPC as a clinical cardioprotective strategy has yet to be fulfilled because of the unpredictable nature of AMI and the need for intervention prior to an ischaemic episode (Hausenloy *et al.*, 2008). However, in scheduled surgical interventions such as elective coronary artery bypass graft, or in unstable angina which often precedes AMI, the ischaemic episode can be predicted and this cardioprotective strategy could be of greater clinical benefit.

1.6.2 Pharmacological preconditioning

Ischaemic preconditioning can also be mimicked by perfusing the heart with drugs in the place of brief ischaemic episodes, and is termed pharmacological preconditioning. Pharmacological preconditioning has been observed with many agents including acetylcholine (Critz *et al.*, 2005), bradykinin (Critz *et al.*, 2005), opioids (Peart *et al.*, 2005), and volatile anaesthetics (Bienengraeber *et al.*, 2005). Pharmacological preconditioning is thought to occur via a similar mechanism to IPC.

1.6.3 Differences in kinase recruitment between IPC and pharmacological IPC

Despite ischaemic preconditioning and pharmacological preconditioning producing ultimately the same cardioprotective effect through activation of the RISK pathway and inhibition of the mPTP, there appear to be some differences between the recruited components of the RISK pathway. For example ischaemic preconditioning appears to activate p38 and Erk1/2 at reperfusion, whilst pharmacological preconditioning with isoflurane only causes activation of Erk1/2 (da Silva *et al.*, 2004). In addition TNF- α appears to activate STAT-3 to induce cardioprotection and not through the conventional activation of the RISK pathway via Akt and Erk1/2 activation. These findings imply that there are potentially multiple kinases that may become activated during ischaemic preconditioning, but which all appear to converge on causing inhibition of the mPTP opening at reperfusion. How all of these kinases interact and the exact mechanism of action is currently unknown.

1.7 Ischaemic postconditioning

Ischaemic postconditioning (IPOST) was first reported in 2003 by Zhao *et al.* (2003b) who reported that three, 30 second intermittent periods of left coronary artery occlusion at the onset of reperfusion, after a 60 minute occlusion period, caused significant infarct limitation in dog hearts (44%), comparable to that seen by IPC (40%).

IPOST was an extremely important discovery for several reasons. Firstly it is clinically relevant to patients who sustain an AMI because it is a therapeutic intervention that

can be applied at reperfusion (Hausenloy *et al.*, 2008). Secondly, it increased the research interest in cardioprotective intervention at reperfusion (Vinten-Johansen *et al.*, 2005). Thirdly, there was finally convincing evidence for reperfusion injury being a distinct entity (Yellon *et al.*, 2006). Interestingly, ischaemic pre- and postconditioning are not additive (Tsang *et al.*, 2004), despite there being a significant similarity in the kinases activated by both interventions (Hausenloy *et al.*, 2005b).

1.7.1 Mechanism of action

Several studies investigating the mechanism of action of ischaemic-postconditioning have demonstrated a reduction in oxidative stress (Sun *et al.*, 2005), mitochondrial calcium accumulation (Sun *et al.*, 2005), improved endothelial function (Zhao *et al.*, 2003b) and reduced inflammation (Zhao *et al.*, 2003b). Ischaemic postconditioning has also been shown to confer cardioprotection through the inhibition of mPTP opening (Argaud *et al.*, 2005; Bopassa *et al.*, 2006), in a similar fashion to ischaemic preconditioning. The involvement of the RISK pathway in pharmacological postconditioning was first demonstrated in 2002 by Schulman *et al.* (2002), by demonstrating that administration of urocortin at the time of reperfusion protected the intact heart from reperfusion injury both *in vitro* and *in vivo*, thorough the up-regulation of the Erk1/2-dependent signalling pathway. They proposed that the hearts possessed a pro-survival reperfusion injury salvage kinase pathway that had the potential to be exploited by pharmacological agents to protect the myocardium against lethal reperfusion injury. Subsequently from these findings the concept extended beyond the classical RISK pathway (P13k/Akt and ERK) to include other pro-survival kinases such as PKG, PKC- ϵ , ps70S6K and GSK-3 β .

A recent publication by Skyschally *et al.* (2009) questioned the activation of the RISK pathway in postconditioning in an *in vivo* pig study. They observed an increase in RISK pathway protein phosphorylation at reperfusion in both postconditioned and normally reperfused hearts, despite a reduction in infarct size. In addition inhibition of PI3K with wortmanin did not block the cardioprotective effect of postconditioning.

There still remains some controversy about the use of ischaemic postconditioning as a cardioprotective strategy, as several research groups have reported the inability to reproduce data (Dow *et al.*, 2007; Schwartz *et al.*, 2006). It has been suggested that ischaemic postconditioning is only effective if the preceding ischaemic period does not exceed 45 minutes in rodents (Tang *et al.*, 2006b). Postconditioning is clearly a more recent discovery and investigation into the mechanism of action and application of this potential cardioprotective strategy are still evolving.

1.7.2 Clinical intervention with ischaemic-postconditioning

There have been several small scale clinical trials conducted to investigate the potential cardioprotective effect of ischaemic postconditioning. This involves using an invasive technique in which several cycles of low pressure coronary angioplasty balloon inflation and deflations are performed in the coronary artery immediately following functional reperfusion. It has been demonstrated that ischaemic-postconditioning is cardioprotective in patients undergoing primary percutaneous coronary intervention for MI, measured by a reduction in infarct size, attenuated ST segment elevation, and improved coronary blood flow and myocardial reperfusion (Laskey, 2005; Ma *et al.*, 2006; Staat *et al.*, 2005). A recent study by Thibault *et al.* (2008) showed that IPOST produced a persistent reduction in infarct size and improved long-term functional recovery in patients with acute myocardial infarction. These findings provide much promise for a post-ischaemic cardioprotective intervention.

1.7.3 Differences in kinase recruitment between IPC and IPOST

Various studies investigating the key components of the cardioprotective signalling pathways have provided convincing evidence to show that most, if not all, of those involved in early reperfusion in the post-conditioned heart are the same as those observed during the trigger phase of IPC (Penna *et al.*, 2006a; Penna *et al.*, 2006b; Tsang *et al.*, 2004; Yang *et al.*, 2005b; Yang *et al.*, 2004). Perhaps the most profound evidence is the observation that combining IPC and IPOST resulted in no additional cardioprotection (Yang *et al.*, 2004). However, connexin-43 has recently been shown

to be an essential feature of IPC, but not required for IPOST (Heusch *et al.*, 2006; Schulz *et al.*, 2007).

Chapter 1 Part II

1.8 Endogenous gaseous mediators

Since the discovery that gases can be endogenously produced by living cells, gaseous mediators have received increasing interest over the past two decades as biological mediators. Gases have a special advantage as biologically active mediators because being small molecules they can infiltrate the 3-dimensional structure of receptors, enzymes and channels and alter their chemical structure to affect biological function (Tang *et al.*, 2006a). Nitric oxide (NO) and carbon monoxide (CO) have previously been identified as gaseous mediators (Cao *et al.*, 2001; Furchgott *et al.*, 1980; Wang, 1998). Hydrogen sulphide (H₂S) is commonly recognised and described as a gas with an offensive odour of rotten eggs (Petersen, 1977). The toxicology of H₂S has received the greatest attention in the biological literature, dating from the 1713 treatise *De Morbis Artificum Diatriba* (Treatise On the Diseases of Workers) by the Italian physician Bernardino Ramazzini (Lambert *et al.*, 2006) who described the irritant and toxic effects of the gas in occupational exposure. A physiological role of H₂S in mammalian tissues was first proposed in 1989 when endogenous levels were detected in the rat brain (Warenycia *et al.*, 1989) and normal human post-mortem tissues (Goodwin *et al.*, 1989). Since this discovery H₂S has received increasing interest and is now regarded along with CO and NO as an endogenous gaseous mediator produced by regulated pathways and exerting discrete physiological actions (Wang, 2002). The recent discovery of endogenous sulphur dioxide (SO₂) (Du *et al.*, 2008), demonstrates just one of many potential endogenous gaseous mediators that are yet to be discovered.

1.8.1 Classification criteria for a gaseous mediator:

The following criteria have been proposed for a substance to qualify as a gaseous mediator (Wang, 2003):

1. It is a small molecules of gas
2. It is freely permeable to membranes. As a result their effects do not rely on cognate membrane receptors, and they have endocrine, paracrine and autocrine effects
3. It is endogenously and enzymatically generated and regulated
4. It has well defined and specific functions at physiologically relevant concentrations
5. Its cellular effects may or may not be mediated by second messengers, but should have specific targets

H₂S appears to satisfy these criteria, justifying its classification as a gaseous mediator.

1.9 Biochemistry of H₂S

1.9.1 Biosynthesis of H₂S

H₂S is a reactive thiol with strong reducing activities. It is produced predominantly by the tissue-specific enzymes that regulate the metabolic pathways for sulphur-containing amino acids, principally methionine. The two key enzymes are cystathionine- β -synthase (CBS) or cystathionine- γ -lyase (CSE). Both are pyroxidal-5'-phosphate-dependent (P-5'-P) enzymes, that use L-cysteine, derived from the dietary amino acid methionine, as their principal substrate (Hosoki *et al.*, 1997). Catabolism of methionine-derived homocysteine to L-cysteine occurs in cardiovascular cells and tissues as well as in the liver (Chen *et al.*, 1999) through the transsulfuration pathway which is initiated by CBS (as summarised in Figure 1.5). This catalyses a β -replacement exchange reaction between the hydroxyl group of serine and the thiolate of homocysteine to form cystathionine which is converted to L-cysteine and α -ketobutyrate by CSE (Chen *et al.*, 1999). L-cysteine then forms the endogenous substrate for H₂S production. A third H₂S synthesizing enzyme, 3-

mercaptosulfurtransferase (MST), also exists (Tang *et al.*, 2006a). The initial stage of H₂S production involving MST involves the conversion of L-cysteine to 3-mercaptopyruvate by cysteine aminotransferase (Stipanuk *et al.*, 1982).

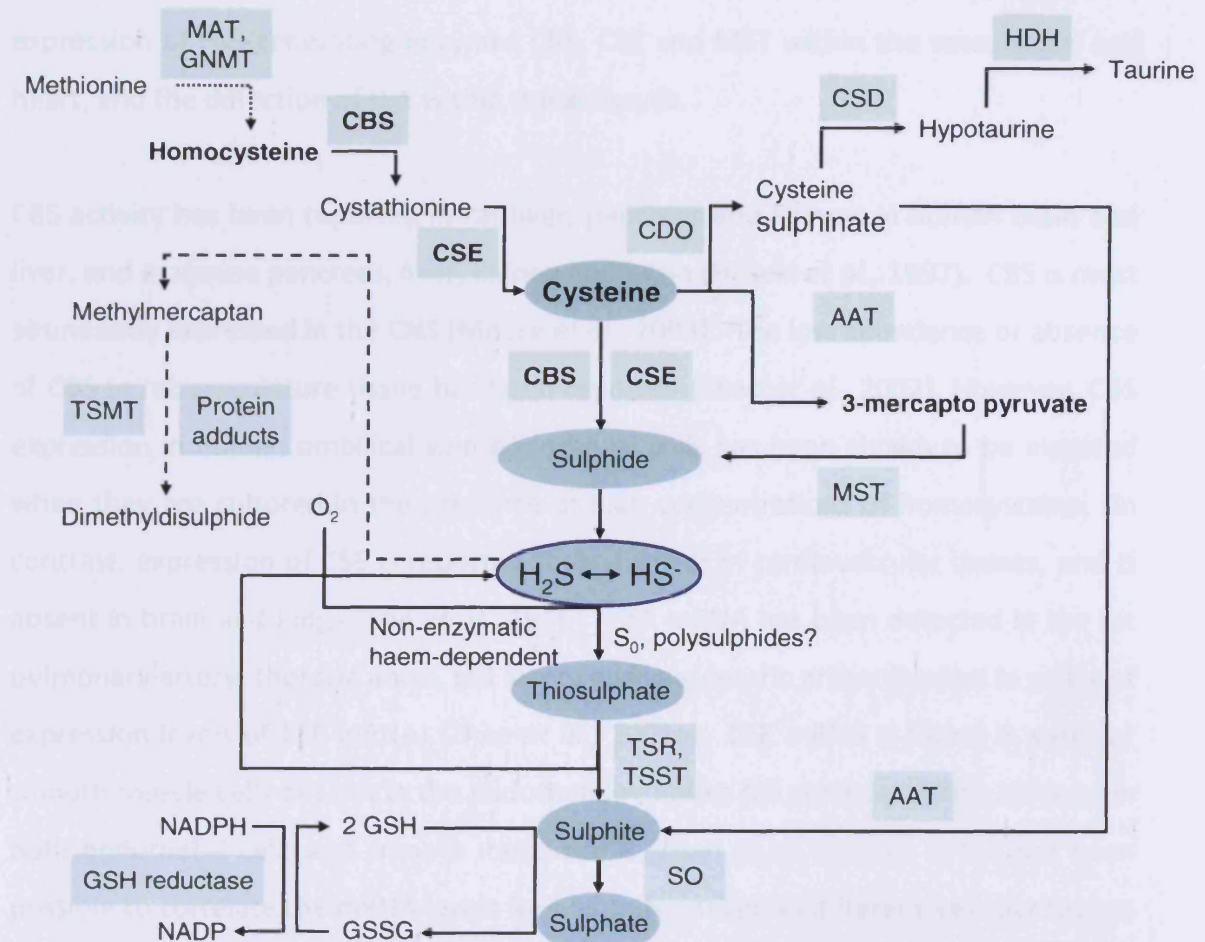


Figure 1.5 The primary enzymatic route of H_2S production uses the tissue specific enzymes cystathionine- γ -lyase (CSE) and cystathionine- β -synthase (CBS) using L-cysteine as substrate. In mitochondria cysteine can be converted to 3-mercaptopyruvate forming the substrate for H_2S production using mercaptopyruvate sulphur transferase (MPST) enzyme. MAT = methionine adenosyltransferase, GNMT = glycine N-methyltransferase, CDO = cysteine deoxygenase, CSD = cysteine dehydrogenase, AAT = aspartate aminotransferase, TSR = thiosulphate sulphur, TSST = thiosulphate sulphur transferase, SO = sulphite oxidase, GSH = glutathione, TSMT = thiol-S-methyltransferase, S_0 = elemental sulphur.

1.9.2 Localisation and expression of H₂S synthesising enzymes

There is strong evidence to suggest that the cardiovascular system is an endogenous source of H₂S generation (Hosoki *et al.*, 1997). This evidence includes both the expression of H₂S-generating enzymes CBS, CSE and MST within the vasculature and heart, and the detection of H₂S within these tissues.

CBS activity has been reported in rat liver, pancreas and kidney, in human brain and liver, and in mouse pancreas, liver, kidney and brain (Hosoki *et al.*, 1997). CBS is most abundantly expressed in the CNS (Moore *et al.*, 2003). The low abundance or absence of CBS in rat vasculature tissue has been reported (Zhao *et al.*, 2002). However, CBS expression in human umbilical vein endothelial cells has been shown to be elevated when they are cultured in the presence of high concentrations of homocysteine. In contrast, expression of CSE is reported to be highest in cardiovascular tissues, and is absent in brain and lungs (Abe *et al.*, 1996). CSE mRNA has been detected in the rat pulmonary artery, thoracic aorta, tail artery and mesenteric artery (ranked in order of expression levels of CSE mRNA) (Zhao *et al.*, 2001a). CSE mRNA is found in vascular smooth muscle cells but not in the endothelium, unlike NO synthase which is found in both endothelial cells and smooth muscle cells (Zhao *et al.*, 2001a). It has not been possible to correlate the mRNA levels for CSE to H₂S levels in different vascular tissues because the protein expression of CSE has not been determined (Wang, 2003), although it has been shown that vascular tissues produce measurable amounts of H₂S (Hosoki *et al.*, 1997). CSE is also present in the heart.

MST receives relatively little coverage in the literature although its presence has been reported in the proximal tubular epithelium in the kidney, pericentral hepatocytes in the liver, cardiac tissue, and in the brain (Griffith, 1987). The MST pathway is reported to contribute to H₂S production in the myocardium (Griffith, 1987; Swaroop *et al.*, 1992). In mitochondria, MST can produce H₂S from 3-mercaptopyruvate or oxidise its sulphur to sulphite and thiosulphate. In the cytosol, thiocysteine formed by CSE can act as an acceptor of the sulphur transferred from 3-mercaptopyruvate by MST (Tang *et al.*, 2006a).

1.9.3 Physiological concentrations and metabolism of H₂S

H₂S is detectable within blood and many tissues (see Figure 1.6). In some reports, concentrations of up to 160 µM in brain and 100 µM in blood are quoted. Although the liver has a high H₂S-generating capacity and may be responsible for maintaining the concentration of H₂S in the blood, 100 µM H₂S would be readily detectable by the human nose while, clearly, blood does not normally smell of free H₂S. There is some uncertainty about the reliability of H₂S assay methods and the fact that total sulphate and sulphite has been measured in some studies, rather than free H₂S, may account for this apparent anomaly. However, concentrations of unoxidised H₂S are detectable although likely to be far lower than initially estimated.

Organ/location	Species	H ₂ S conc. (µM)	Reference
Blood	Rat	10	(Mason <i>et al.</i> , 1978)
	Human	10 – 100	(Richardson <i>et al.</i> , 2000)
Plasma	Rat	46	(Mason <i>et al.</i> , 1978)
	Human	160	(Abe <i>et al.</i> , 1996)
Brain	Rat	50 – 160	(Goodwin <i>et al.</i> , 1989) (Warenycia <i>et al.</i> , 1989)
	Human		
	Bovine		

Figure 1.6 Summary of the endogenous tissue concentrations of H₂S in rat, bovine, and human. Note that all the concentrations are in the micro-molar range, which would be readily detectable by the human nose, but despite this human blood does not normally smell of free H₂S.

Intracellular and tissue concentrations of H₂S appear to be regulated by a number of metabolic pathways. Metabolism is principally via oxidation in mitochondria, or by cytosolic methylation. H₂S can also be scavenged by methaemoglobin, metallo- or disulphide-containing molecules such as oxidised glutathione (Beauchamp *et al.*,

1984). H_2S is excreted mainly by the kidney as free or conjugated sulphate (Beauchamp *et al.*, 1984).

In LPS induced endotoxic shock in the rat, inhibition of H_2S synthesis lowers ALT levels, which is a specific marker for hepatic parenchymal injury in the liver (Collin *et al.*, 2005). This was implied by Zhao *et al.* (2003a), who also showed that the production of H_2S in the rat liver was greater than in the vascular tissue, suggesting that the liver may be responsible for maintaining the concentration of H_2S in circulation.

1.9.4 Toxicity of H_2S :

The threshold of detection of H_2S by the human nose is between 0.02 and 0.13 ppm (Costigan, 2003). 50% of the human population are able to detect 0.005 ppm. At concentrations greater than 100 ppm olfactory fatigue can occur, suggesting that odour perception is unreliable as a warning of high exposure to H_2S (Costigan, 2003). In humans a single short term exposure to H_2S greater than 500 ppm can be fatal (Costigan, 2003). Exposure for a few minutes to concentrations of 1000 ppm and above are likely to cause rapid unconsciousness and death (Costigan, 2003). At high concentrations of H_2S , death is caused by depression of the respiratory centres, whilst at lower concentrations, death is due to pulmonary oedema and congestion (Costigan, 2003). It is estimated that H_2S poisoning accounts for 30 deaths per year.

The reported toxic level of H_2S is less than two fold greater than the endogenous levels reported in rat brains (Warenycia *et al.*, 1989). This suggests that there must be a steep dose-response relationship of H_2S in the physiological concentration range before it becomes toxic (Zhao *et al.*, 2001a). Therefore there must be a fine homeostatic control mechanism to regulate H_2S levels at a physiological level (Wang 2003). The LD_{50} for H_2S in the rat has been reported as 15mg/kg (192 $\mu\text{mol/kg}$) (Warenycia *et al.*, 1989).

Acute intoxication of H_2S results in a loss of central respiratory drive (Costigan, 2003). H_2S acts by complexing with the Fe^{3+} of mitochondrial cytochrome oxidase resulting in

the blockade of oxidative metabolism. It may also target carbonic anhydrase (Mangani *et al.*, 1992) and monamine oxidase (Warenycia *et al.*, 1989).

1.10 H₂S interaction with NO

A specific sulphide receptor has not been identified, but H₂S is known to interact with other biological mediators and signal transduction components to produce its effects in the cardiovascular system. It has been reported that there is an interaction between NO and H₂S, since the production of both gases can be increased by pro-inflammatory mediators in haemorrhagic shock (Mok *et al.*, 2004) and bacterial lipopolysaccharide (Li *et al.*, 2005). However, the precise relationships between H₂S and NO and the physiological significance of their interactions are far from clear. H₂S has been shown to both enhance (Zhao *et al.*, 2002) and attenuate (Hosoki *et al.*, 1997) the relaxant effect of NO in the rat aorta, whilst NO has been shown to enhance the release of H₂S in rat vascular tissues and increase the expression of CSE in cultured vascular smooth muscle cells (Zhao *et al.*, 2001a). There is evidence that NO and peroxynitrite react with H₂S to form a novel nitrosothiol, which has been proposed to regulate the physiological effects of NO and H₂S (Whiteman *et al.*, 2006).

In homogenates of rat aorta, NO donors increased CSE-dependent H₂S generation in a cGMP-dependent manner (Zhao *et al.*, 2003a). Prolonged incubation of cultured vascular smooth muscle cells with NO donors produced an increase in CSE mRNA and protein levels (Zhao *et al.*, 2001a). Conversely, circulating H₂S levels, CSE gene expression and enzymatic activity in the cardiovascular system were reduced in rats chronically treated with an NO synthase inhibitor, highlighting the physiological significance of NO in the regulation of H₂S production in the cardiovascular system (Lowicka *et al.*, 2007). Interestingly H₂S (50-200 µM) has been shown to inhibit NO production and down regulate inducible NO synthase expression in lipopolysaccharide-stimulated macrophages via a mechanism that involves heme oxygenase expression and CO production (Oh *et al.*, 2006). The H₂S donor compound NaHS has also been shown to inhibit the activity of eNOS in a concentration dependent manner, in a biochemical assay using recombinant bovine eNOS (Kubo *et al.*, 2007).

As well as NO regulating H₂S generation, endogenous H₂S has been proposed to have a role in the regulation of vascular NO production (Ali *et al.*, 2006). Combining NaHS with NO donor compounds inhibited the vasorelaxant effect of acetylcholine and histamine in rat aortic rings. Intravenous infusion of NaHS in anaesthetised rats caused a significant increase in mean arterial blood flow, which was reduced in the presence of the NO synthase inhibitor L-NAME. These findings suggest that H₂S and NO react together under physiological conditions to form a product that has little or no vascular activity *in vitro* or *in vivo*. This molecule may be a nitrothiol as previously proposed by Whiteman *et al.* (2006). Therefore, it is conceivable that a major physiological role of H₂S in the vasculature may not be to act as a vasodilator, but rather to regulate local concentrations and activity of NO. Furthermore, the observation that concentrations of H₂S (200 µM) greater than those found physiologically (50-160 µM) are needed to produce a vasodilator response (Ali *et al.*, 2006), supports a regulatory role of native H₂S in the vasculature, as opposed to a direct vasodilator effect.

1.11 General physiology of H₂S in cardiovascular tissues

1.11.1 Regulation of vascular tone

Hosoki *et al.* (1997) showed that exogenous H₂S produced concentration-dependent relaxations of rat thoracic aorta and portal vein and guinea pig ileum. Zhao *et al.* (2001a) subsequently reported that intravenous bolus injections of H₂S caused a transient decrease in the blood pressure of rats. This hypotensive effect was mimicked by the K_{ATP} channel opener pinacidil and blocked by glibenclamide, a K_{ATP} channel blocker. Since heart rate was not significantly affected, the *in vivo* action of H₂S was believed to be specific to vascular smooth muscle. Exogenous H₂S caused relaxation of precontracted rat isolated aortic rings. These effects were again blocked by glibenclamide and mimicked by pinacidil confirming the involvement of K_{ATP} channel opening as a key mechanism of the vasorelaxant effect (Zhao *et al.*, 2001a). However, a recent study investigating the effect of H₂S in aortic rings has questioned the role of K_{ATP} channels (Kubo *et al.*, 2007) since glibenclamide did not inhibit the actions of H₂S in mouse aortic rings. It is also uncertain whether endogenous H₂S is a

vasorelaxant under normal physiological conditions, since the vascular effect of H₂S is dependent on tissue concentration. There is also evidence that H₂S promotes rapid vasoconstriction in well-oxygenated isolated vessel models (Koenitzer *et al.*, 2007). Ali *et al.* (2006) have also shown that concentrations of NaHS (10-100 µM) that cause constriction of aortic rings are attenuated after removal of endothelial cells, suggesting an indirect effect of H₂S on vascular smooth muscle cells. Concentrations of NaHS greater than 100 µM resulted in aortic relaxation. This dual vasodilator and vasoconstrictor effect of H₂S has also been observed in human intestinal mammary artery. These apparently contradictory findings are consistent with the notion that there may be a role of H₂S to quench NO when applied at low concentrations, resulting in constriction. There also appears to be a time dependent effect of NaHS administration which determines its effective concentration.

H₂S donors in animals produce a transient hypotensive effect, whilst pharmacological inhibitors of enzymatic H₂S production have no haemodynamic effect (Zhao *et al.*, 2001a). This is in distinction to NO synthesis inhibitors which cause a significant vasoconstrictor effect in normal animals and increase peripheral vascular resistance (Koenitzer *et al.*, 2007). These findings suggest that under normal physiological conditions H₂S may not be directly responsible for regulating blood pressure but does so via its interaction with the NO pathway (Ali *et al.*, 2006). However, Yang *et al.* (2008) recently showed that CSE knock-out mice exhibited pronounced hypertension and diminished endothelium dependent vasorelaxation, suggesting that H₂S may in fact be a physiological vasodilator and regulator of vascular blood pressure.

In disease states, it is possible that alterations of H₂S generation play a more direct role in influencing vascular tone. In lung tissue from rats with experimentally induced hypoxic pulmonary hypertension and in *in vivo* hypertensive rats, a reduction in CSE expression and activity was seen (Chunyu *et al.*, 2003; Zhong *et al.*, 2003). Parenteral administration of H₂S opposed a rise in pulmonary arterial pressure and in part prevented pulmonary vascular remodelling, identified by the thickness of the vascular wall in isolated rat lung (Chunyu *et al.*, 2003). This implies that H₂S deficiency might be related to pulmonary vasoconstriction and intimal thickening which are

pathological factors associated with pulmonary hypertension (Moore *et al.*, 2003). In rats, the NO synthase inhibitor L-NAME produced a time dependent elevation in systolic blood pressure associated with inhibition of CSE gene expression and H₂S production. Exogenous H₂S prevented the development of hypertension induced by L-NAME (Zhong *et al.*, 2003).

There is also the possibility that excessive levels of H₂S could contribute to hypotension associated with either sepsis or endotoxaemia. In endotoxin-treated rat arteries there was significant elevation of H₂S production (Hui *et al.*, 2003). Endogenous H₂S has been shown to play a role in haemorrhagic shock in the rat (Mok *et al.*, 2004). In rats, withdrawal of blood induced haemorrhagic shock with profound lowering of arterial blood pressure, associated with a transient increase in plasma H₂S levels beyond 60 minutes. Inhibitors of CSE produced a partial restoration of the lowered arterial blood pressure in a time-dependent manner. Pre-treatment with a CSE inhibitor increased the arterial pressure after 60 minutes, suggesting that H₂S does not have a role in the immediate response to haemorrhagic shock, but is involved in the later stages (Mok *et al.*, 2004). Liver CSE mRNA was also significantly increased after 60 minutes (Mok *et al.*, 2004). However, the cells/tissues responsible for increased synthesis of H₂S after blood withdrawal are unknown. Therefore further studies are required to identify the cell types involved in H₂S synthesis following haemorrhagic shock.

1.11.2 Effects on angiogenesis

Cai *et al.* (2007) reported a pro-angiogenic role of H₂S and a potential for the involvement of the prosurvival PI3K/Akt signalling pathway. The pro-angiogenic effect of H₂S (10-20 µM) was observed in an *in vitro* endothelial cell culture assay. The ability of H₂S to promote proliferation, adhesion, migration, and tube-like structure formation in endothelial cultures was observed. The PI3K inhibitor LY294002, inhibited the pro-angiogenic effects of H₂S in endothelial cells. Interestingly, NaHS was also shown to increase Akt phosphorylation, which was inhibited in the presence of the PI3K inhibitors LY294002 and wortmanin. These observations suggest that H₂S may illicit its pro-angiogenic effect via PI3K/Akt signalling. A pro-angiogenic role of

H₂S, at physiologically relevant concentrations (10-50 μ mol/kg/day), was also demonstrated in an *in vivo* mouse matrigel plug assay (Cai *et al.*, 2007). There appears to be no other published work to date on this potentially novel therapeutic approach.

1.11.3 Vascular smooth muscle cell proliferation

S-diclofenac, a novel H₂S releasing derivative of a non-steroidal anti-inflammatory drug (NSAID), has been shown to inhibit aortic smooth muscle cell proliferation (Baskar *et al.*, 2008). The anti-apoptotic effect of H₂S in smooth muscle cells has also been demonstrated using NaHS (Sivarajah *et al.*, 2008). A mechanism of action involving opening of K_{ATP} channels has been proposed (Sivarajah *et al.*, 2008). The anti-apoptotic effect of H₂S may be of importance for the prevention of cell proliferation in vascular occlusive disorders and prevent atherosclerosis, vascular graft occlusion and restenosis after angioplasty (Sivarajah *et al.*, 2008). Interestingly, endogenously produced H₂S has also been shown to have pro-apoptotic effects in human aortic smooth muscle cells over-expressing the H₂S synthesising enzyme CSE (Yang *et al.*, 2005a). The difference in findings between these studies may be as a result of differences in experimental protocols, or most likely the concentration of H₂S. H₂S has also been shown to concentration dependently suppress the proliferation of smooth muscle cells through the mitogen activated protein kinase (ERK1/2) pathway (Du *et al.*, 2002).

1.11.4 Inotropic and chronotropic effects

In the heart, NaHS had a concentration-dependent negative inotropic effect in both isolated perfused rat hearts and *in vivo* rat models (Geng *et al.*, 2004c). These effects were partially blocked in the presence of glibenclamide, suggesting a role of K_{ATP} channel opening in mediating the inotropic effect of H₂S. This is consistent with the negative inotropic effects of other K_{ATP} channel activators which induce cell membrane hyperpolarisation. K_{ATP} activation can also result in blockade of L-type calcium channels, resulting in reduced calcium entry and myocardial contractility (Cole *et al.*, 1991). The effect of H₂S on isolated cardiac myocyte contractility and electrically-induced Ca²⁺ transients were investigated during β -adrenergic stimulation.

NaHS significantly attenuated the effects of isoproterenol and forskolin. NaHS also reversed isoproterenol-induced cAMP elevation and forskolin-stimulated adenylyl cyclase activity. These findings suggest that H₂S may antagonise negative consequences of sympathetic over activation by generating negative feedback to cAMP production (Yong *et al.*, 2008b).

NaHS has been shown to exert a concentration-dependent negative chronotropic effect in rat isolated hearts (Ji *et al.*, 2008). This action has also been observed in mice receiving 1 mg/kg NaHS at reperfusion in an *in vivo* model (Elrod *et al.*, 2007). NaHS also caused a concentration-dependent reduction in the firing rate of rabbit pacemaker cells in sinoatrial nodes. This negative chronotropic effect was blocked in the presence of glibenclamide, again implicating an increase in potassium efflux through the K_{ATP} channels to mediate the effect in pacemaker cells (Xu *et al.*, 2008).

Two emerging areas of H₂S biology that may have particular pathophysiological and therapeutic applications will now be discussed.

1.12 Cytoprotective actions of H₂S in myocardial ischaemia/reperfusion injury

During the last 20 years, a number of endogenous mediators have been observed to play key roles in determining the cellular responses to ischaemia/reperfusion, attenuating the processes of irreversible injury that result in cell death, as described in Part I. A number of these mediators, or chemical derivatives of them participated in IPC and/or IPOST (Ferdinandy *et al.*, 2007). Recent evidence suggests that H₂S may exert preconditioning and postconditioning cytoprotective actions. Geng *et al.* (2004a) showed that H₂S could be endogenously generated in the heart as a physiological cardiac regulator protecting cardiac function. In rat hearts subjected to isoprenaline-induced necrosis, exogenous H₂S caused a reduction in mortality and improved cardiac function (Geng *et al.*, 2004a). Johansen *et al.* (2006) provided the first evidence that exogenous H₂S can limit infarct size induced by ischaemia/reperfusion in the rat heart in a concentration-dependent manner. It was shown that the K_{ATP} channel blockers glibenclamide or sodium 5-hydroxydecanoate (mitochondrial K_{ATP} channel selective blocker) attenuated the protective effect of NaHS, supporting the

involvement of K_{ATP} channel opening in the protective mechanism of action. Bian *et al.* (2006) further reported that exogenous H_2S (33 μM) reduced the incidence and severity of ischaemia/reperfusion arrhythmias in the isolated rat heart.

The evidence summarised above suggests that exogenously administered H_2S is protective. There is however a discordance in the literature regarding the role of H_2S in ischaemic preconditioning. Bian *et al.* (2006), Pan *et al.* (2006), and Hu *et al.* (2008) showed endogenous H_2S to play a protective role, whilst Siverajah *et al.* (2006) showed no involvement of endogenous H_2S . Whether endogenously-generated H_2S plays a role in the natural phenomenon of ischaemic preconditioning is an unresolved issue.

1.13 Pro- and anti-inflammatory actions of H_2S

There is currently considerable interest in the roles of H_2S as a mediator in inflammation. The majority of *in vivo* models imply that H_2S is a pro-inflammatory mediator (Lowicka *et al.*, 2007). For example it has been reported that the plasma H_2S concentration, and CSE expression and activity are increased in lipopolysaccharide-treated mice (Li *et al.*, 2005) and in a caecal ligation and puncture model of sepsis in the mouse (Zhang *et al.*, 2006). The findings of the latter study imply that H_2S enhances the inflammatory response and end organ damage associated with sepsis. For example, in a mouse lung and liver model of sepsis, the CSE inhibitor compound PAG was shown to attenuate the inflammatory response, indicated by a reduction in myeloperoxidase activity, a marker of neutrophil infiltration. PAG was also shown to reduce mortality after caecal ligation and puncture (Zhang *et al.*, 2006). The mechanism through which H_2S exerts these pro-inflammatory actions *in vivo* is unclear.

Data from *in vitro* models are more ambiguous and support anti-inflammatory actions of H_2S . H_2S donors have been shown to inhibit aspirin-induced leucocyte adhesion to the endothelium of rat mesenteric venules, whilst inhibitors of H_2S synthesis caused an increased adhesion of leucocytes (Zanardo *et al.*, 2006). CSE expression has also been shown to be up-regulated by LPS and pro-inflammatory cytokines (Nagai *et al.*,

2004), which could account for the increased H₂S production during inflammation. Overproduction of H₂S during inflammation may be detrimental by enhancing the inflammatory response and associated tissue damage. For example fMLP-activated neutrophils are able to non-enzymatically convert H₂S to sulphite in an NADPH and ROS dependent manner (Mariggio *et al.*, 1997). An increased concentration of serum sulphite was observed in LPS-treated rats (Mitsuhashi *et al.*, 1998) and in patients with pneumonia (Mitsuhashi *et al.*, 2004). Sulphite is highly toxic, despite low levels having an important bactericidal action. Sulphite stimulates the production and release of ROS from neutrophils (Labbe *et al.*, 1998) and the adhesion of neutrophils to the endothelium (Shigehara *et al.*, 2002). It can also react with peroxynitrite to form toxic sulphur radicals (Reist *et al.*, 1998). Therefore there appears to be a paradoxical role of H₂S in inflammation, because whilst a small increase in H₂S production enhances non-specific host defence, an excessive concentration may result in inflammation and tissue damage. This may explain the differences in role of H₂S in inflammation observed between models. It is also important to note that increased H₂S concentrations in a given disease state may not be deleterious. It is still unclear if it is the increased H₂S that is responsible for the pathogenesis of disease or if H₂S is elevated in an attempt to counteract the pathogenic agent.

H₂S has been shown to up-regulate anti-inflammatory and cytoprotective genes including heme-oxygenase in pulmonary smooth muscle cells *in vivo* (Oh *et al.*, 2006), and macrophages *in vitro* (Qingyou *et al.*, 2004). The up-regulation of heme-oxygenase is thought to occur downstream of ERK activation and results in the production of CO, a well characterised cytoprotectant and anti-inflammatory gas (Ryter *et al.*, 2006).

In the past decade, interest in H₂S as an endogenously-produced biological mediator has broadened research in this field considerably beyond its toxicology, resulting in an exponential increase in the annual number of published reports since 1995. The range of body systems and cell types extends beyond cardiovascular effects of H₂S and it seems likely that this field of investigation is set to expand. There are still a number of unanswered questions surrounding its physiological and pathophysiological

relevance and roles, notably relating to specific molecular targets of H₂S and mechanisms of action. Indeed it is feasible that as a highly reactive thiol, native or unconjugated H₂S reacts with numerous biological targets and elicits actions through multiple non-specific mechanisms.

1.14 Overview and scope of the work presented in this thesis:

Current findings from World Health Organisation predict that as far as 2030, cardiovascular disease will remain the leading cause of mortality in the world (Mathers *et al.*, 2006), there is a need for more effective solutions to protect patients suffering from acute myocardial infarction. Due to the unpredictable nature of the onset of acute myocardial infarction, clinical therapies should ideally be aimed at the time of reperfusion when flow is restored to the heart and minimise reperfusion injury. The recent discovery of the RISK pathway, and its expanding components and activators, provides the basis for a greater understanding of the cardioprotective mechanisms that occur at reperfusion and various algorithms that can be used to prime this pathway, therefore suggesting a novel target for cardioprotection. The increased interest in H₂S over the past decade has led to its discovery in the myocardium and also revealed the potential cardioprotective effect of exogenous H₂S, suggesting a potential endogenous mediator of cardioprotection. However, there is only a weak characterisation of the enzymes that synthesise H₂S in the myocardium and the effect of ischaemia-reperfusion on their ability to function. As yet a pharmacological target for H₂S has not been identified or a specific mechanism of action determined.

Major points of focus:

The aim of the studies described in this thesis was to characterise H₂S synthesis in the myocardium and elucidate potential cardioprotective roles of H₂S during ischaemia-reperfusion.

The general hypothesis underpinning this work was that H₂S synthesising enzymes exist in the myocardium and the resulting H₂S provides cardioprotection against ischaemia-reperfusion.

The specific questions addressed are:

1. Does the rat myocardium contain H₂S synthesising enzymes and is there a predominant isoform?
2. What effect does ischaemia-reperfusion have on the ability of the rat myocardium to synthesise H₂S?
3. Does exogenous or endogenously stimulated H₂S protect the rat myocardium against regional ischaemia-reperfusion injury?
4. Does H₂S activate the RISK pathway to induce cardioprotection in the rat myocardium?

The approach to these scientific questions involved using a variety of experimental techniques and rat myocardium. The techniques undertaken included PCR and Western blotting to identify the presence of the mRNA and protein for the H₂S synthesising enzymes in rat myocardium (Chapter 3). Cloning of the H₂S synthesising enzymes and transfection into cell lines, to validate the specificity of a custom synthesised antibody to CSE used for Western blotting studies. Isolated perfused rat hearts were used to investigate the effect of regional ischaemia-reperfusion and observe the effects of exogenous and endogenously stimulated H₂S on infarct size limitation (Chapter 4). The isolated perfused hearts were also used to decipher the mechanism of cardioprotection elicited by H₂S by use of pharmacological tools to inhibit components of the RISK pathway (Chapter 6). Biochemical assays were also performed to measure the tissue concentration of H₂S and also determine the maximal H₂S synthesising capacity of exogenously stimulated enzymes and observe the effect of ischaemia and reperfusion (Chapter 5).

Chapter 2: General methods

2.1 Langendorff perfused isolated rat hearts

2.1.1 Isolated perfused rat hearts according to Oscar Langendorff

In 1897 Oscar Langendorff developed his isolated perfused mammalian heart preparation (Skrzypiec-Spring *et al.*, 2007). Prior to his discovery, the idea of perfusing mammalian hearts had been briefly investigated by Carl Ludwig (Zimmer, 1998). However their model was of limited success because the hearts being perfused were empty, beating, non-ejecting hearts and were of limited physiological value. Isolated frog hearts had also been perfused, a technique discovered by Elias Cyon in 1866 (Zimmer, 1998), but these hearts lacked coronary arteries and again this model was of limited physiological value. Langendorff's technique was unique because he was able to maintain perfusion of hearts for several hours in a model that mimicked the physiological flow of blood through the heart (Zimmer, 1998). Langendorff was the first to demonstrate that the coronary arteries are responsible for the delivery of oxygen and nutrients to the heart and the cardiac mechanical function is reflected by changes in coronary circulation (Taegtmeyer, 1995). He was also able to confirm previous findings relating to the negative chronotropic effect of both vagal nerve stimulation and administration of muscarinic agonists, demonstrated the positive chronotropic effect of atropine, and also discovered that potassium chloride arrests the heart (Taegtmeyer, 1995).

Despite many modifications over the years, the principles of Langendorff's isolated perfused hearts remains the same (Sutherland *et al.*, 2000). Essentially, hearts are cannulated via the ascending aorta and perfused with either blood or a crystalloid buffer. The force of the perfusate forces the aortic valve shut, resulting in retrograde flow of the perfusate through the coronary arteries via the ostia at the aortic root. The perfusate then drains into the right atrium and exits the heart via the coronary sinus (Sutherland *et al.*, 2000).

The Langendorff isolated perfused heart model has been used widely in cardiovascular research over the past century due to its ability to produce highly reproducible data, relative low cost, and the ability to measure a wide range of experimental parameters.

2.1.2 Animal species for use in Langendorff isolated heart perfusion

The Langendorff technique is suitable for any mammalian heart, although Langendorff himself originally used cat hearts as well as hearts obtained from dogs and rabbits (Zimmer, 1998). The rat is now the most commonly used heart donor animal and is the most characterised (Sutherland *et al.*, 2000). More recently, with the emergence of knock-out mice the mouse heart has started to receive increased interest (Sutherland *et al.*, 2000). The only limitation with the rat heart is its short action potential (Hearse *et al.*, 2000). Guinea pig hearts can also be perfused, but cannot be used for experiments investigating regional ischaemia. This is owing to their extensive collateral vessels (Sutherland *et al.*, 2000), which make the task of inducing regional ischaemia, via reversibly tying the left main descending coronary artery, impossible. Guinea pigs do however have a role in global ischaemic experiments where all the flow to the heart can be stopped.

2.1.3 Application of the Langendorff isolated perfused heart preparation

The Langendorff isolated perfused heart preparation can be used for a variety of cardiovascular studies with various parameters measured. A common application of this model is to measure infarct sizes in hearts subjected to ischaemia-reperfusion and assess the infarct limitations of potential cardioprotective or cardiotoxic drugs. Ischaemia can either be induced regionally by occluding flow through the left anterior descending coronary artery, or regional ischaemia or low flow ischaemia can be induced by either stopping or reducing flow respectively (Sutherland *et al.*, 2000). Ischaemia can also be induced to pre- or post-condition the heart and the protective effect observed. Other studies include accessing arrhythmia scores as an indication of cardioprotection, as well as accessing the underlying cause of arrhythmias (Skrzypiec-Spring *et al.*, 2007). The effects of Hypoxia and anoxia can be investigated by altering the oxygen content of the perfusate, which in an *in vivo* model may compromise the survival of the animal (Skrzypiec-Spring *et al.*, 2007). The model can also be used to prepare heart samples for protein analysis using Western blotting and quantifying proteins of interest after various treatment protocols.

2.1.4 Perfusion algorithms

There are two algorithms for perfusing isolated hearts, depending on the experimental parameters to be measured. A fixed pressure system is achieved by using a constant hydrostatic pressure of the perfusate. This is achieved by maintaining the volume of perfusate at a fixed height to generate the desired pressure. The advantage of this setup is that is more physiologically relevant since *in vivo* the mean perfusion pressure is maintained in the intact coronary circulation (Skrzypiec-Spring *et al.*, 2007). A constant flow system can also be used which utilises a mechanical pump to deliver the perfusate the heart. This ensures that a constant amount of perfusate is delivered to the heart irrespective of the physiological functioning of the heart. This enables coronary perfusion pressure changes to be monitored and used as an index of the coronary vascular resistance. The main disadvantage of this system being the autoregulatory mechanisms of coronary arteries being overridden and the absence of shear mediated endothelium-derived relaxing factor production (Sutherland *et al.*, 2000). However, its advantages include: the increased ability to measure coronary perfusion pressure with a pressure transducer than monitor coronary flow, the vasoactive properties of drugs can be easily observed, and the constant flow ensures that the concentration of drug that reaches the heart is constant even if vasoactive drugs are used (Skrzypiec-Spring *et al.*, 2007). Since the studies conducted in this thesis are concerned with the infarct size limitation during ischaemia-reperfusion a constant pressure system was used, since it is thought to be the most physiologically relevant in relation to ischaemia (Doring, 1990). The constant flow system overrides the autoregulatory mechanisms, resulting in the amount of perfusate delivered to the heart not being physiologically altered in response to changes in heart rate or contraction during ischaemia (Skrzypiec-Spring *et al.*, 2007).

2.1.5 Limitations of the model

As with any animal model used to mimic human physiology there are limitations and the Langendorff isolated heart perfusion is without exception. The main disadvantage owing to the fact the heart is in isolation and therefore receives no neuronal input from the autonomic and central nervous systems, as well as no influence from other

organs (Skrzypiec-Spring *et al.*, 2007). This however, may be classed as an advantage since it allows direct actions of drugs to be observed on the heart in the absence of peripheral influences. As with any experimental model, the isolated heart is also a constantly deteriorating preparation, with an observable run-down in function over the course of the experiment. However, hearts maintain sufficient function for experiments of three hours or longer to be conducted. The robustness and reproducibility of the data obtained by the Langendorff isolated perfused heart preparation far outweigh the disadvantages and make for a very reliable model for investigating a variety of cardiovascular studies.

2.2 Setup of Langendorff isolated perfused hearts

2.2.1 Excision of rat heart

Male Sprague Dawley rats (270-350) were terminally anaesthetized with a mixture of sodium pentobarbitone (60 mg/kg) and heparin (100 units) i.p. Barbiturates are the anaesthesia of choice because they can be easily administered and have minimal depressive action on the functioning of the heart. Once the animal was under a suitable level of anaesthesia, as determined by lack of a pedal withdrawal reflex, a transabdominal incision was made to expose the xiphoid process and the diaphragm. A bilateral incision along the rib cage opened the thorax. The heart was exposed by moving the thoracic cage upwards. Once the heart was exposed it was gently cradled in the hand and excised to ensure a good length of aorta was obtained. The excised heart was then immediately arrested in ice cold modified Krebs-Henseleit buffer.

2.2.2 Cannulation and perfusion of the isolated rat heart

The excised cold arrested heart was carefully removed from the Krebs buffer using forceps and the aortic root gently opened and inserted over a plastic cannula. The aorta was tied to the cannula using three 3-0 silk ties, and retrogradely perfused with modified Krebs-Henseleit buffer (NaCl 118.5 mM, NaCO₃ 24.8 mM, d-Glucose 11 mM, KCl 4.7 mM, MgSO₄·7H₂O 1.2 mM, KH₂PO₄ 1.2 mM, CaCl₂·2H₂O 2.25 mM) at a constant perfusion pressure of 74 mmHg produced at 100 cm of water. Initially, whilst cannulating the aorta, the flow was reduced to aid the process, before being returned

to full flow once the heart was in situ. Care was taken to ensure that the heart was orientated so that the ostium of the left anterior descending coronary artery was facing forward, enabling a 3-0 silk suture (Ethicon, UK) was passed around the left anterior descending coronary artery close to its origin near the left atrial appendage. A snare was formed by passing the ends of the suture through a small plastic tube. The snare was left un-tightened until regional ischaemia was induced at the appropriate time in the experiment. The left atria was cut away to reveal the entrance to the left ventricle. A fluid filled latex balloon was inserted into the left ventricle and left ventricular end-diastolic pressure set between 5 and 10 mmHg. The left ventricular diastolic and systolic pressures were measured at timed intervals throughout the experiment, by use of a pressure transducer connected to a PowerLab running Chart (Version 4.0). From these parameters the developed pressure in the heart could be calculated. The heart rate was calculated by the PowerLab data acquisition system by counting the number of left ventricular peaks per second which is then transposed into beats per minute (bpm). Coronary flow rate was measured by timed collection of the coronary effluent over a 30 second period and expressed as flow rate in mls per minute. Throughout the experiment the Krebs buffer was maintained at a constant 37 °C to ensure that the heart was kept normothermic. This was achieved by a thermostatically controlled water circulator continually pumping water, containing 0.5% copper sulphate to kill bacteria, through a jacketed pre-warmer column and a Baker coil. This ensured that as the Krebs buffer flowed from the reservoir, passed through the pre-warmer jacket, and Baker coil before entering the cannula and subsequently the heart, it was maintained at 37 °C. The heart was also immersed in a heart jacket also connected to the circulator that ensured it remained normothermic especially during regional ischaemia when flow was reduced. The reservoirs containing the Krebs buffer were also continually bubbled with carbogen (95% O₂ and 5% CO₂), to ensure that the pH of the Krebs buffer remained at 7.4 and to oxygenate the myocardial tissue. A K-type temperature probe, connected to a digital thermometer, was inserted into the pulmonary sinus to enable the internal temperature of the heart to be monitored during the experiment. A diagram of the setup is shown in figure 2.1 and heart canulation shown in detail in figure 2.2.

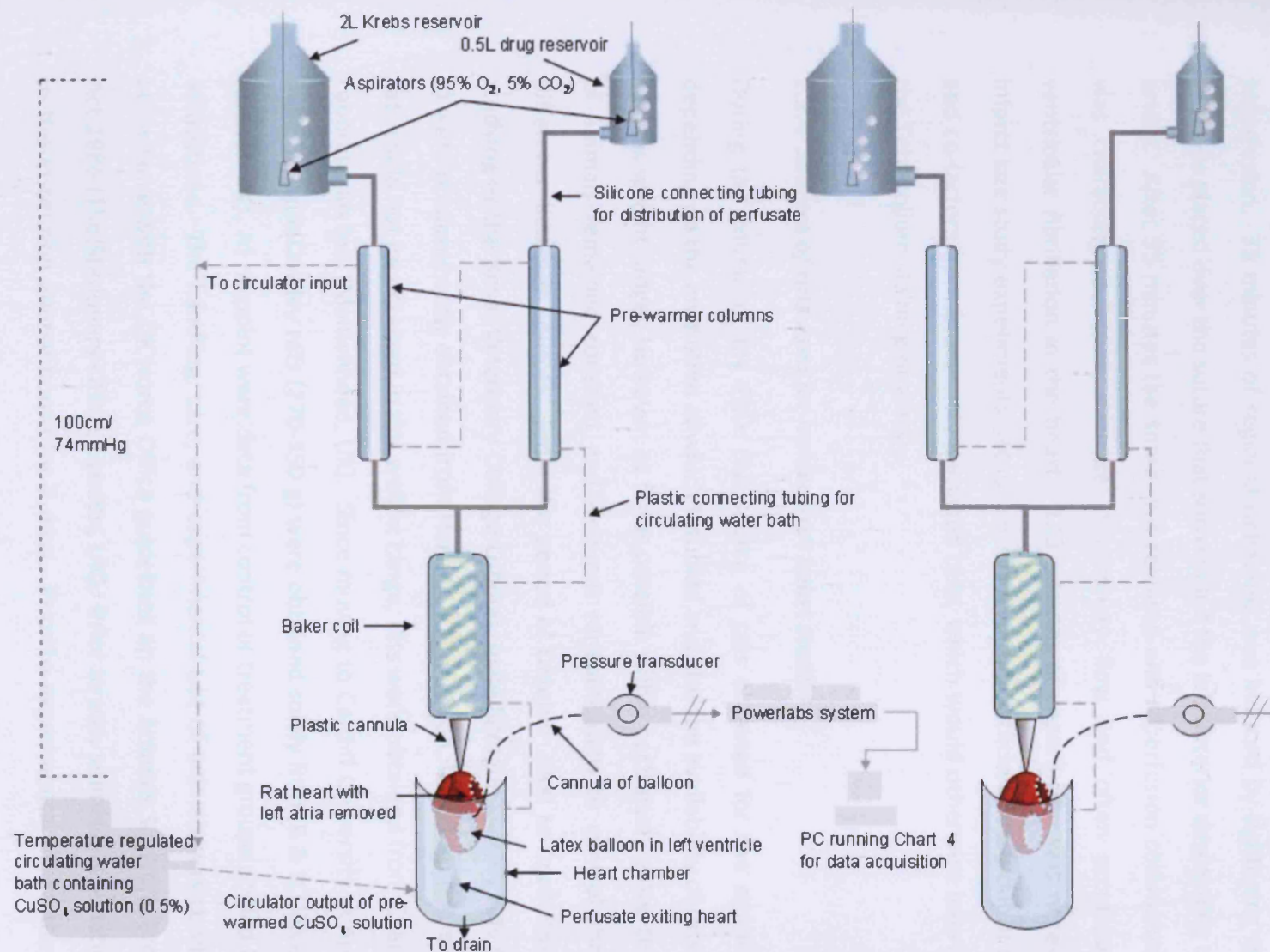


Figure 2.1 Summary diagram of the Langendorff constant pressure perfused isolated rat heart. Hearts were randomised between left and right rigs to ensure no bias was placed on the preparations. Note that although not shown in the diagram, the pre-warmer columns, Baker coils, and heart jackets are connected in parallel to ensure even distribution of pre-warmed water and constant temperature of Krebs perfusate.

2.2.3 Treatment groups

Hearts were randomised to treatment groups to ensure no bias was placed on the study. The specific treatment protocols for each series of experiments are outlined in the subsequent experimental chapters. In all studies where infarct limitation was the end point of the study, the hearts received a stabilisation period ranging from 20-30 minutes, followed by 35 minutes of regional ischaemia, and 120 minutes of reperfusion. 35 minutes of regional ischaemia was induced by tightening the snare that was placed over the suture that surrounded the left anterior descending coronary artery. After 35 minutes the snare was released and reperfusion commenced. This was characterised by an increase in coronary flow and often accompanied by ventricular fibrillation in the heart. 120 minutes of reperfusion was necessary for infarct size study experiments because it ensured that sufficient wash-out of enzymes and co-factors from irreversibly damaged cells, which would otherwise interfere with the Tetrazolium staining process.

2.2.4 Sources of rats used for isolated rat heart studies

During the course of my PhD, the source of rats obtained for the studies varied depending on the institution at which I studied and also the availability of rats within a specific weight range. However, as far as possible within each study group the source of animals remained constant and frequent randomisation to control showed no difference between sources. From the period of October 2006 to April 2007, whilst studying at the Royal Veterinary College London, male Sprague Dawley rats (270-350 g) were predominantly obtained from Harlan UK Ltd. (Oxon, UK). In the instance that rats could not be obtained in this weight range, rats were obtained from Charles River Laboratories Inc. (Maidenhead, UK). Since moving to Cardiff University in April 2007, male Sprague Dawley rats (270-350 g) were obtained solely from B & K Universal Ltd. (Bristol, UK). At no point were data from control or treatment groups pooled between institutions. The handling, care, and experimental use of animals was conducted in accordance with the UK Home Office guidelines on the Animals Scientific Procedures Act 1986 (The Stationary Office, London, UK). Prior to use, animals were acclimatised in the institution animal house for 7 days. Prior to experimental usage the animals

were subjected to 12 hour light-dark cycles and received chow, containing 4% fat and 18% protein, and water ad lib. On the day of experimentation rats were housed in transportation boxes for minimal time periods before being sacrificed.

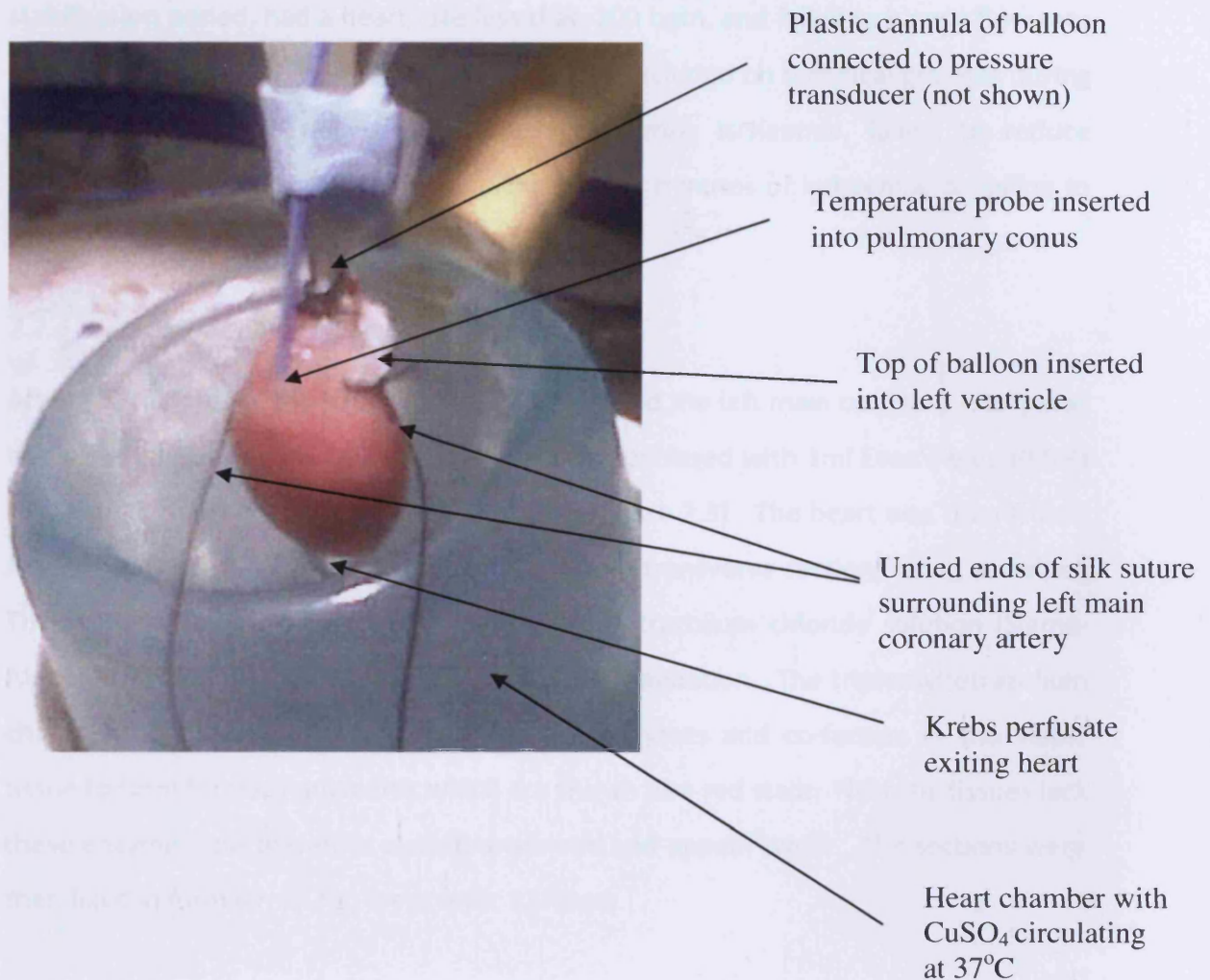


Figure 2.2 Close-up image of the cannulated heart. After removal of the left atria a latex balloon is inserted into the left ventricle. The balloon allows a basal diastolic pressure to be set and allows developed pressure within the ventricle to be measured as well as the heart rate calculated. The Krebs perfusate, seen exiting the heart, is measured by timed collection to determine the flow rate. The silk suture passed around the left main coronary artery can be tightened with snare to induce regional ischaemia. Release of the snare allows reperfusion to occur.

2.2.5 Exclusion criteria

To ensure that data was as reproducible within treatment groups and not biased between groups, strict exclusion criteria were used. Hearts were excluded from the study if they failed to obtain a developed pressure less than 50 mmHg during the stabilisation period, had a heart rate less than 200 bpm, and failed to have a flow rate of between 10 and 15 ml/min. Hearts were also excluded on technical grounds during experiments such as pulling the suture out during ischaemia, failing to reduce coronary flow by at least a third during the first 5 minutes of ischaemia, or failing to restore coronary flow at reperfusion.

2.2.6 Determination of infarct size

After 120 minutes of reperfusion, the suture around the left main coronary artery was tied off using a surgeons knot and the heart was perfused with 1ml Evan's blue (0.5%) to stain the non-ischaemic tissue (as shown in figure 2.3). The heart was then frozen for 24 hours at -20 °C and then cut into 5, 2 mm transverse sections using a scalpel. The sections were incubated in a 1% triphenyltetrazolium chloride solution (Sigma-Aldrich, UK) at 37°C for 20 minutes with frequent agitation. The triphenyltetrazolium chloride solution reacts with dehydrogenase enzymes and co-factors in the viable tissue to form formazin pigments which are shown as a red stain. Necrotic tissues lack these enzymes and therefore remain unstained and appear white. The sections were then fixed in formalin (3.7%) for at least 12 hours.

After fixation, the heart sections were scanned, being held in place with a transparent ruler which allowed calibration of the image analysis software. The use of the image analysis software ImageJ 1.38x (National Institute of Health, USA) enabled the three regions in each heart section to be digitally analysed using planimetry. During infarct analysis the blue region represented the normally perfused tissue, the red represented the risk zone, and the white unstained tissue represented the infarcted tissue (as shown in figure 2.3). The volumes of the regions were calculated by multiplying the areas by 0.2 cm (the thickness of the tissue sections). The infarct size was expressed as a percentage of the risk zone. The larger the infarct size the more

severe the irreversible damage to the myocardium. Infarct sizes in hearts were measured blind, to ensure that no bias was placed on specific treatment groups.

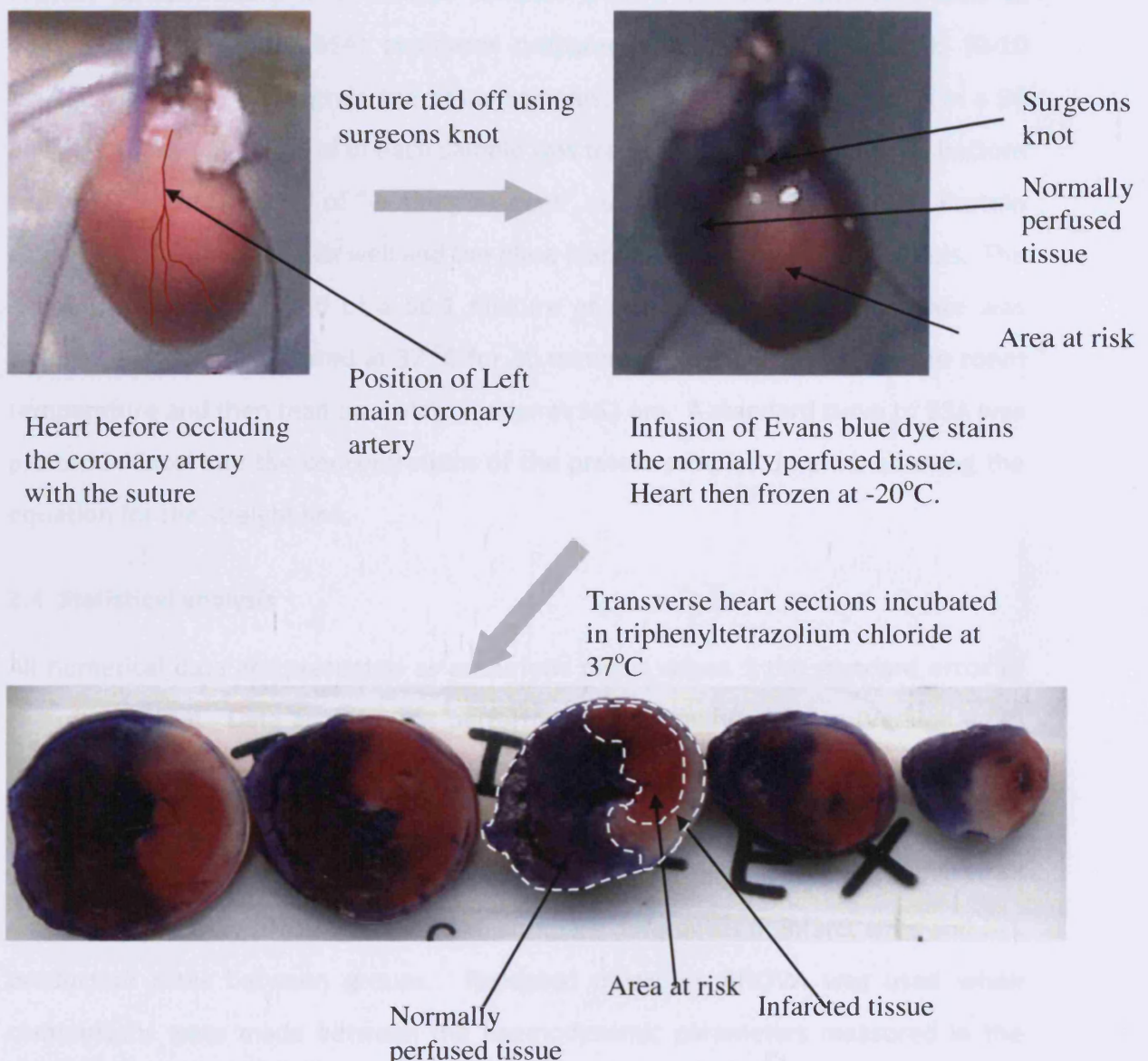


Figure 2.3 Preparation of Evans blue and triphenyltetrazolium chloride double stained transverse heart sections. The blue region represents the normally perfused tissue,, the red represents the area at risk, and the white unstained tissue represents the infarcted necrotic tissue. The regions would be traced round and measured using planimetry software ImageJ. Volumes of the zones are calculated by multiplying the areas by the thickness of the sections (0.2 cm and average between the 5 sections. A ruler is also scanned with the sections to calibrate the ImageJ software.

2.3 Protein determination of tissue samples

The BCA (bicinchoninic acid) protein assay was performed to enable the colorimetric detection and quantification of total protein to be determined in the tissue samples. Protein concentrations in the tissue samples were determined with reference to bovine albumin serum (BSA) standards prepared at known concentrations (0-10 mg/ml) and assayed alongside the tissue samples. The assay was performed in a 96 well flat bottom plate. 10 μ l of each sample was transferred into a 96 well flat bottom plate in duplicate. 200 μ l of "working reagent", supplied in the Pierce BCATM Protein assay kit, was added to each well and the plate placed on a shaker for 30 seconds. The working reagent consisted of a 50:1 mixture of solution A and B. The plate was covered in foil and incubated at 37 °C for 30 minutes. The plate was cooled to room temperature and then read on a plate reader at 562 nm. A standard curve to BSA was plotted in Excel and the concentrations of the protein samples determined using the equation for the straight line.

2.4 Statistical analysis

All numerical data are presented as arithmetic mean values \pm the standard error of the mean (SEM). Data analysis was performed using GraphPad Prism (Version 4.00). Analysis of variance (ANOVA) was used to compare the mean values of different groups. A test for normality for each data set was performed using the Kolmogorov-Smirnov test prior to ANOVA calculations. Normal distribution was observed in all data sets. One-way ANOVA was used to compare differences in infarct sizes and H₂S production rates between groups. Repeated measures ANOVA was used when comparisons were made between the haemodynamic parameters measured in the Langendorff model. In all cases post hoc analysis was performed using a Newman-Keuls test. The probability (p) level of 5% ($p < 0.05$) was defined as the level at which data was said to be significantly different and was indicated on graphs by the use of the standard star notation system (*). P values obtained from the statistical tests that was < 0.01 were said to be highly significant and shown on graphs by the use of the standard star notation system (**).

Chapter 3 Characterisation of the H₂S synthesising enzymes cystathionine- γ -lyase (CSE) and cystathionine- β -synthase (CBS) within the rat heart

3.1 Introduction

It was reported in Chapter 1, that CSE was the predominant H₂S synthesising enzyme in the myocardium (Abe *et al.*, 1996; Geng *et al.*, 2004c). This statement has been supported by numerous published studies reporting the detection of CSE mRNA using PCR (Abe *et al.*, 1996; Geng *et al.*, 2004c; Wu *et al.*, 2006; Yang *et al.*, 2006; Zhao *et al.*, 2001a; Zhong *et al.*, 2009; Zhong *et al.*, 2003; Zhu *et al.*, 2007). Whilst the detection of mRNA does suggest the potential for a tissue to express CSE protein, it does not necessarily mean that the tissue will. At the time of performing the Western blotting studies, there were no published studies of CSE protein expression in the myocardium. This is surprising given the extensive claims in the literature that the CSE protein predominates in the myocardium. The lack of a commercially available antibody probably accounts for this and also raises questionability into the accuracy of the published studies. It therefore appears that there is lack of in depth characterisation of the mRNA and protein expression of CSE in the myocardium, and in particular in different regions of the heart. The experiments performed in this chapter were an attempt to provide robust evidence for whether CSE is the predominant H₂S synthesising enzyme in the myocardium through the use of both PCR to detect mRNA, and a custom synthesised antibody to CSE to detect protein. In addition CBS mRNA and protein expression was also explored in the myocardium to complete the characterisation.

3.1.1 Polymerase chain reaction (PCR)

PCR is a commonly used technique for the detection of DNA for a specific gene of interest, which was first introduced by Mullis in 1983 (1992). The technique relies on the use of specific primers, which are short sequences of around 20 bases that are complimentary to a sequence of DNA on the gene of interest. Both forward (3'-5') and reverse (5'-3') primers are used which enables a specific DNA sequence, usually up to 10kb, of interest to be amplified. The PCR reaction involves using a thermocycler to repeatedly heat and cool the PCR reaction mix, enabling denaturation of the DNA strands, annealing of the primers and extension of the sequence to occur, each time this sequence of events occurs it is called a cycle.

Typically between 20 and 40 cycles are performed in a PCR reaction. The PCR reaction mix consists of a DNA template that contains the sequence of interest. Typically this can be produced from tissue by the extraction of mRNA and then converting it to cDNA in a reaction. The reaction mix also contains the forward and reverse primers that are complimentary to the DNA sequence of interest, Taq polymerase, Deoxyribonucleotide triphosphates (dNTPs) which are the bases from which the Taq polymerases synthesises a new DNA strand, and a buffer solution that contains magnesium ions. The PCR products can be separated by gel electrophoresis on an agarose gel and the use of DNA ladders enables the produce size to be determined (Bartlett *et al.*, 2003).

3.1.2 Western blotting

Western blotting is a technique used for the detection of a specific protein in a cell lysate by use of the knowledge that a specific antibody will bind to a specific antigen. An antibody is a protein with a unique structure that enables it to bind specifically to a particular substance known as its antigen. This enables a protein of interest to be detected amongst a group of proteins. The term Western Blot was originally coined by Burnette (1981) which was a play on the "Southern Blot" technique name, a DNA detection technique developed by Edwin Southern (1992). Initially proteins are separated by size using SDS-polyacrylamide gel electrophoresis (SDS-PAGE), with the lightest proteins travelling the furthest down the gel. The size separated cells are then transferred from the gel to a nitrocellulose membrane (Towbin *et al.*, 1979), which provides a stable support for the remainder of the technique. The protein of interest can then be detected by incubating the membrane with a specific antibody that can react with the SDS-solubilised protein. The bound antibodies are then revealed using a secondary anti-immunoglobulin antibody bound to a luminescent label. This binds to the primary antibody and the resulting luminescence can be detected on photographic film. Colourimetric, fluorescent, and radioactive techniques can also be employed. The greater amount of protein, the greater the binding of antibody, and the greater the density of the band of light detected. Densitometry can be used to measure the intensity of the

resulting bands and these can be compared between samples in semi-quantitative manner. The use of a ladder marker enables the size of the protein detected to be compared to the expected size so confirm its presence (Blancher *et al.*, 2001).

3.2 Aim

The aim of the studies conducted in this chapter was to characterise the expression of the H₂S synthesising enzymes CSE and CBS within the rat myocardium at both the mRNA and protein level.

3.3 Hypothesis

1. CSE mRNA will be predominantly expressed over CBS in the left and right ventricles of rat myocardium
2. CSE protein will be predominantly expressed over CBS in the left and right ventricles of rat myocardium

3.4 Objectives

The validity of these hypotheses was tested as follows:

1. To determine the mRNA expression levels of CSE and CBS in the rat myocardium using PCR and specific primers
2. To use selective CSE and CBS antibodies and perform Western blotting to determine the relative protein expression levels of the two synthesising enzymes in the rat myocardium
3. To confirm the selectivity of the CSE antibody using cloning and over-expression of the CSE protein in a GH3 cell line

3.5 Methods

3.5.1 PCR

3.5.1.1 Detection of tissue CSE and CBS mRNA using PCR

PCR was performed in tissue left ventricle (LV), right ventricle (RV), atria (A), liver (Li) and cerebellum region of the brain (B) from rats to determine if there was mRNA expression of the CSE and CBS enzymes in the rat heart.

3.5.1.2 Source of primers used

The CSE and CBS primers were designed from gene sequences and purchased from Sigma-Aldrich, UK with a forward and reverse sequence selected for each gene of interest. A BLAST search was performed to check that the primers were intro spanning to ensure that they were specific to the mRNA of interest. The primers were prepared in dH₂O to produce 100 µM stocks and then were diluted a further 1:10 in dH₂O prior to use (10 µM).


```

1 CCGTCCCAGCATGCAGAAGGACGCCTCCTCCAGCGGCTTCTGCCAGCTTCCAGCACTT
61 TGCCACTCAGGCCATCCACGTGGGACCAGAGCCGGAGCAATGGAGTTCGCGTGCTGTGGT
121 GCTGCCCATTTCGCTGGCCACCACGTTCAAACAGGACTCTCCAGGCCAGTCCTCGGGTTT
181 TGTATACAGCCGCTCTGGAAATCCGACGAGGAATTGCTTGGAAAAAGCAGTGGCTGCACT
241 GGATGGGGCAAAGCACTGTTTGACCTTCGCTCGGGGCCTTGCCGCCACCACAACGATTAC
301 CCATCTTTTAAAGCAGGAGATGAAGTCATTTGCATGGATGAAGTGTATGGAGGCACCAA
361 CAGGTACTTCAGGAGGGTGGCATCCGAGTTTGGACTGAAGATTTCTTTGTGGATTGTTT
421 CAAAACCAAATTGCTGGAGGCAGCGATCACACCACAGACCAAGCTTGTTTGGATTGAAAC
481 ACCCACAACCCAACTTGAAGTTGGCCGACATCAAAGCCTGCGCACAATTGTCCACAA
541 ACACAAAGACATCATTCTGGTTGTAGATAACACTTTCATGTCTGCATATTTCCAGAGACC
601 TTTGGCTCTGGGTGCTGATATTTGTATGTGTTCTGCCACAAAATACATGAACGGCCACAG
661 TGATGTTGTATGGGCTTAGTGTCTGTTACTCCGATGACCTCAACGAACGGCTTCGTTT
721 CCTGCAGAATTCTCTCGGGGCAGTTCCTTCTCTTCGATTGTTACCTCTGCTGCCGAGG
781 CCTGAAGACACTGCAGATCCGGATGGAGAAACACTTCAGGAATGGGATGGCAGTGGCCCCG
841 TTTCTGGAGTCTAATCCCCGGGTAGAAAAGGTTATTTATCCTGGGCTACCGTCTCACC
901 TCAGCATGAGCTCGCCAAACGTCAGTGCACGGGTGCCCGGGATGGTCAGTTTCTATAT
961 CAAGGGTACTCTGCAGCATGCTCAGGTCTTCTCAAAAATATAAAGCTGTTTGTCTGGC
1021 TGAGAGCCTGGGAGGATATGAGAGTCTGGCTGAGCTTCCAGCAATCATGACCCATGCCTC
1081 CGTGCCTGAGAAGGACAGAGCTACCCTCGGGATCAGTGACACACTGATCCGACTTCTGT
1141 GGGCTAGAGGATGAAAAGGACCTTCTGAAGACCTGGGTCAAGCTTTAAAGGCAGCGCA
1201 CCCTTAAAGTTTCGAGTCAAAGCCGGCATTCCAGTGTGCCATCAGCAGCAGCAAGG
1261 GGCCAGACCTTCTGAATAACTGGACAGACCATTAAAGGAGCATCTGCAGAACTTCGCAGTG
1321 AACATTTTAAGACCCTAGTGATTTTACAGCTGTAACCTTACAGGGATCTTCCCTTAAGGA
1381 CTGTCTTCTGCTAACAGGTTGTTCTGTTAGTATCATTCTGATAGTTTGTGTATTTGTG
1441 TTCAAGGAAGAGAGTTGTATTATTTGGGGATCATGTTGCTTCTTTTCTTTTCTTT
1501 CTTGGTAGCCTAAGATATGTTTAAATCATGTTTACAAAATTTAGTATTGATGTTTATG
1561 AAGTTAAATTATTCAATGAACGGTCTTAAATCAACTGTAGGGGTTTTTTTTTGAAAAAT
1621 TATTGAAAGTGGGGGGTCTTTATTTAATTACCATAAGCCAAAAAATCAAATATTTGGAA
1681 TATCTACTGTGAAATTCTAGTGATTAAAGGTTGTAAGTACTTGATGTTGTTTTCTTAA
1741 TGG

```

Figure 3.1 CSE primer design showing the region of amplification for forward and reverse primers respectively. Forward primer starts at base 225 and spans 18 bases AAGCAGTGGCTGCACTGG. Reverse primer starts at base 458 and spans 18 bases TCTGTGGTGTGATCGCTG.


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1 TTTCCCGCTCTGAGTCTAGCTGCACCCTGCTCCTGTCTCCCATCCTTGCAAAGCTTGT
61 CTGAGTGGAGCCAACACGCCCAGAGGGGACAGGAGAGTCAACTACTAAACCAACAGGT
121 TCTGCGACCTCAGCAAATCCCAGCATGCCTTCAGGGACATCCCAGTGTGAAGATGGCTCT
181 GCAGGGTGGCCCCAGGACTTGGAGGTACAGCCAGAAAAAGGGCAACTGGAGAAGGGAGCC
241 TCAGGGGACAAGGAAAGAGTCTGGATCTCGCCTGATACCCCAAGCAGATGTACTTGGCAG
301 CTGGGCAGGCCCATGGCGGATTCCCCACATTACCACACAGTGCCGACAAAATCCCCGAAA
361 ATTTTGCCAGATATTCTGAGGAAAATTGGCAACACCCCTATGGTCAGAATCAACAGGATC
421 TCCAAGAATGCAGGACTCAAGTGCGAGCTGTTGGCCAAGTGTGAGTTCTTCAACGCCGT
481 GGGAGTGTGAAGGACCGCATCAGCCTCCGGATGATTGAAGACGCTGAGCGAGCCGGAACC
541 TTGAAGCCCGGAGACACGATCATTGAGCCAATTCTGGCAACACAGGGATCGGGCTGGCT
601 CTGGCAGCTGCTGTGAAGGGCTATCGCTGCATTATCGTGATGCCTGAGAAGATGAGTATG
661 GAGAAGGTGGATGTGCTGCGAGCTCTGGGAGCTGAGATTGTGAGGACGCCACCAACGCC
721 AGATTCTGATTCCCCGAGTCCCACGTAGGAGTGGCATGGCGACTGAAGAACGAAATCCCC
781 AATTCTCACATTCTGGACCAGTACCGCAATGCCAGCAACCCCTTGGCGCACTACGATGAC
841 ACCGCAGAGGAGATCCTGCAGCAGTGCGACGGGAAGGTGGACATGCTGGTGGCTTCAGCA
901 GGCACGGGTGGCACCATCACGGGTATCGCGAGGAAGCTGAAGGAGAAGTGCCAGGTTGT
961 AAAATCATCGGTGTAGATCCCGAGGGGTCCATCCTCGCGAGCCCGAGGAGCTGAACCAG
1021 ACGGAGCAAACAGCCTATGAGGTGGAAGGGATCGGCTACGACTTCATCCCCACCGTCTG
1081 GACAGGGCGGTGGTGGATAGGTGGTTCAAGAGCAATGATGACGATTCTTCGCCTTCGCC
1141 CGCATGCTCATCTCCAGGAGGGACTGCTGTGCGGTGGGAGTTCAGGCAGCGCTATGGCC
1201 GTGGCTGTGAAGGCTGCCAGGAGCTAAAGGAAGGACAGCGCTGTGTGGTCATCTGCC
1261 GACTCTGTGCGCAACTACATGTCCAAGTTCTTGAGTGACAAATGGATGCTGCAGAAAGGC
1321 TTCATGAAGGAGGAGCTCTCCGTGAAGAGACCTGGTGGTGGCATCTGCGTGTCCAAGAG
1381 CTGAGCCTATCAGCACCGCTGACCGTGTGCCACTGTACCTGTGAGCACACCATCGCC
1441 ATCCTCCGGGAGAAGGGTTTTGACCAGGCACCTGTGGTCAACGAGTCTGGGGCCATCCTA
1501 GGGATGGTGACTCTCGGGAACATGTTGTCTCCCTGCTTGTGTTGGAAGGTGCGGCCATCA
1561 GACGAAGTCTGCAAAGTCTCTACAAGCAGTTCAAGCCGATCCACCTGACCGACACACTG
1621 GGATGCTCTCCACATCCTGGAGATGGACCACTTCGCCCTGGTGGTCCATGAGCAGATC
1681 CAATACCGCAACAATGGCGTGTCCAGCAAGCAGCTGATGGTGTGTTGGTGTGTACCGCC
1741 ATTGACCTGCTAAACTTCGTGGCAGCCCGTGAGCAGACCCGGAATAGAGTTCAGAAGTC
1801 AGGACTGGCTTCCATCCTCCCTGCTGGGACTTCTTGGCTTTCAGAGACACCGACTGGTTT
1861 CCACACCCAAGTCCAGCAGGTGGCTGCTGAGGCCAGCACCTCCCTCCTAACGCTCAGC
1921 TCCCTATAGGAATCCTCTATGTCCGAGTAGCTTACGTGGGCTTTCCTCTGGTGTCCAGA
1981 ACCAAGGAATGGCAGCCAGGAAAAGATAGGCACAGACTACACTCGCCACAAGACTCAGGGT
2041 GCCTAGGAAAGTGTCTCTCCAGAGAGGGGCTCCAGCCTGAGAAAGGGCAAACCTGGACT
2101 GACTGTGCTATCCTCAGGGGGCAGTGTGCCCCAGCAAGGGAGCATGTGGGTTTTAA
2161 TGAAGGTGCGTTCAGTGACCTGAGACCCACAGCTGTGAAGTAAACGTCGTGCCTGTACG
2221 GAGTGTACCACTGGGTCATGACCTGCTTAGCAGTTCCTCCTCACATCTCCCTCCTT
2281 CCCGACAAGCACCTACTTTCTGTCTCAACTCTTCTATAAATGAATCACATACCTGTGGT
2341 CATGTCTACCTAATTTTCTGGAATGAGTGCCTTGTGTTTCTGTATGGCCTGTGTCA
2401 GAGTTTCGTTCTTTCCATGGCTGAGGGAGCCATTGTGTGGATGGGTGGTCTTATTTT
2461 CTGGGCATCGGTATGCTTGAATACATTTCCCGCTGGGGTGTAGCCCGGTTGGGCTCACT
2521 GCAAAAAAAAAAAAAAAAAA

```

Figure 3.2 CBS primer design showing the region of amplification for forward and reverse primers respectively. Forward primer starts base 567 and spans 18 bases GCCAACTTCTGGCAACAC. Reverse primer starts at base 891 and spans 19 bases CACCAGCATGTCCACCTTC.

Primers for the two house-keeping genes GAPDH and L19 were also used. GAPDH (glyceraldehyde-3-phosphate dehydrogenase), codes for an enzyme that is vital to the glycolytic pathway. These genes are known to be constitutively expressed in all cell types. Since housekeeping genes are constitutively active, they produce a constant level of mRNA. They are therefore often used as a loading control to verify that identical amounts of sample were added. Since equal volumes of cDNA as opposed to equal concentrations were loaded, the housekeeping genes can be used to test the integrity of the cDNA. They also enabled a comparison of the expression to be made across different tissues, although the type of PCR conducted was not quantitative. However, the expression level of house-keeping genes may vary among tissues or cells and may change under certain circumstances. Therefore, by selecting two house-keeping genes there is a reduced probability of both varying and proving a more accurate quality control.

3.5.1.3 Sample preparation

Tissue samples from 6 male Sprague Dawley rats (270-350 g) were taken after terminal anesthesia with an i.p. injection of a mixture of sodium pentobarbitone (65 mg/kg) and heparin (100 units). The heart was excised and dissected, separating the left and right ventricles, and the left and right atria. 6 samples were taken from the left ventricle, 4 samples from the right ventricle and the pooled left and right atria formed a single sample. The central lobe of the liver was removed and divided into 6 samples. The head of the rat was removed and the brain carefully dissected. The cerebellum was identified and divided into 3 samples. The remainder of the brain was divided into 3 sections. The samples were washed in sterile filtered PBS and blot dried on tissue. The samples were placed in individual 0.5 ml sterile Eppendorf tubes and immediately snap frozen in liquid nitrogen. The samples were stored at -80°C until required for experimentation.

3.5.1.4 Extraction of RNA

30 mg of left ventricle, right ventricle, liver and brain (cerebellum), previously snap frozen in liquid nitrogen, were crushed using a dry ice cooled mortar and pestle. The

resulting powder was transferred into 2 ml Eppendorf tubes. RNA was extracted from the tissue samples using a Qiagen Rneasy Mini kit. An optional on-column DNase digestion with an RNase-free DNase kit was performed during the RNA extraction. Cardiac tissue is far more fibrous than the liver and brain, therefore a proteinase K digest stage was required at the start of the RNA extraction protocol to remove these proteins. After this stage the protocol for RNA extraction was the same as that used for the liver and brain tissue. A total of 50 µl of RNA solution was eluted for each tissue sample. A 10µl aliquot was taken and frozen at -20 °C, so that it could be run on a gel to check for the presence of RNA. 5 µl of loading dye was added to each of the 10µl samples so that it could be run on a 1.6% agarose gel using electrophoresis and the presence of mRNA bands at 18s and 28s detected. The remainder of the RNA sample was frozen at -80°C.

3.5.1.5 Synthesis of cDNA

An Amersham Biosciences first-strand cDNA synthesis kit was used for the generation of full-length cDNA from the RNA templates prepared from the tissue samples. Due to the expense of the kit and large number of samples to process, the volumes of components in the first-strand reaction mix were slightly altered from those suggested by the manufacturer. 7 µl of Bulk first-strand reaction mix was added, along with 1µl of the first strand primer Not I-d(T)₁₈, 1µl DTT solution, 12 µl RNA and 21 µl First volume first-strand reaction. The Not I-d(T)₁₈ primer initially received a 1:25 dilution in dH₂O prior to the addition to a first strand reaction mixture. The results however, remained unaffected.

3.5.1.6 PCR reaction

PCR was performed using the cDNA synthesised from the rat tissue samples. Primers for the CSE and CBS enzymes were selected from those previously published in the literature (Zhao *et al.*, 2001a). For detection of CSE mRNA the forward sequence used was 5'-AAGCAGTGGCTGCACTGG-3', and the reverse primer sequence used was 5'-TCTGTGGTGTGATCGCTG-3'. The PCR reaction consisted of 35 cycles of 95 °C for 30 seconds to denature, 51 °C for 30 seconds for annealing and 72 °C for 1 minute

for extension. A final extension period of 72 °C for 10 minute was added at the end of the 35 cycles (PCR conditions summarised in figure 3.3). The PCR product size was 234 bp. For detection of CBS mRNA the forward sequence used was 5'-GCCAACTTCTGGCAACAC -3', and the reverse primer sequence used was 5'-CACCAGCATGTCCACCTTC -3'. The PCR reaction consisted of 35 cycles of 95 °C for 30 seconds to denature, 55.4 °C for 30 seconds for annealing and 72 °C for 1 minute for extension. A final extension period of 72 °C for 10 minute was added at the end of the 35 cycles (PCR conditions summarised in figure 3.3). The PCR product size was 325 bp. The PCR conditions were determined by entering the primer sequences and product sizes into the PCR thermal cycler (G-storm). Each sample contained 15 µl of the master mix (see Appendix 3 for composition) plus 5µl of respective cDNA in an autoclaved sterile 200 µl PCR tube. House keeping genes, GAPDH and L19, were tested alongside the tissue sample to ensure the reactions were successful. At the end of the PCR reaction cycles, the products were removed and 5µl of loading dye was added to each of the samples (see Appendix 3 for composition).

Primer	Melting	Annealing	Extension	Final extension
CSE	30" @ 95°C	30" @ 51°C	1' @ 72°C	10' @ 72°C
CBS	30" @ 95°C	30" @ 55.4°C	1' @ 72°C	10' @ 72°C

Figure 3.3 Summary of the PCR conditions for the CSE and CBS primers. Each sample underwent 37 cycles of melting, annealing and extension, before a final 10 minute extension step.

10 µl of each of the samples were run on a 1.6% agarose gel (see Appendix 3 for composition), with the addition of a 1 kb and 2-log marker, to ensure that the products were in the correct position. The remainder of the sample was stored at -20 °C.

3.5.1.7 Negative controls

Negative control samples were prepared from the tissue samples of one of the rats used in this study. In the PCR reaction mix a DEPEC water was used in the place of

cDNA, to ensure that the water used to prepare and dilute the constituents of the PCR reaction mix was not contaminated. Also in the PCR reaction mix, cDNA was substituted with RNA for each of the tissue samples to confirm the absence of genomic DNA. These samples underwent the PCR reaction alongside experimental samples. At the end of the PCR reaction cycles, the products were removed and 5 µl of loading dye was added to each of the samples. 10 µl of each of the samples were run on a 1.6% agarose gel, with the addition of a 2-log marker. The remainder of the sample was stored at -20°C. The absence of contamination of the DEPEC water and RNA with genomic DNA was confirmed by the lack of bands in these sample lanes (as shown in figure 3.4).

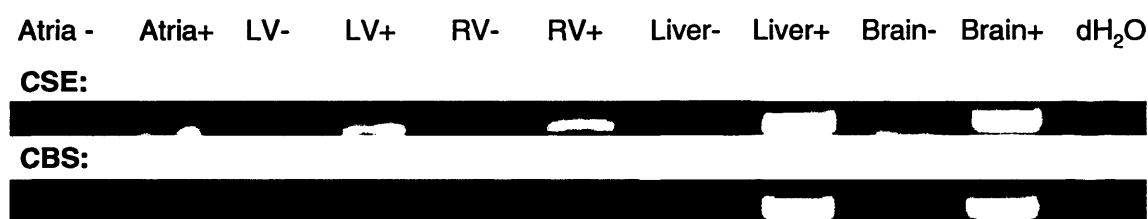


Figure 3.4 *Negative controls for PCR. RNA (-) and DEPEC H₂O (dH₂O) were used in the place of cDNA in the PCR reaction mix, to ensure that the water and RNA used wasn't contaminated with genomic DNA which could falsify the results.*

3.5.1.8 Sequencing of products

The products from the PCR reactions were sequenced in order to confirm that they were the CSE and CBS enzymes that were being detected. Initially the PCR products were run on a new agarose gel (1.6%), but with a blank lane in between each sample. The chosen DNA band was excised with a scalpel under ultraviolet light. DNA extraction was achieved using a QIAquick Gel extraction kit. The sample was frozen at -20 °C prior to being sequenced by Kathy Dominy (RVC, London).

The samples were sequenced using an ABI 3100 Genetic Analyzer and a BigDye® Terminator v3.1 Cycle Sequencing Kit. A BLAST search was performed to confirm the presence of the CSE and CBS DNA in the gel extracted PCR samples.

3.6 Western Blotting

3.6.1 Detection of CSE and CBS protein expression using Western blotting

Western blotting was used to determine if there was protein expression of the enzymes CSE and CBS enzymes in the rat heart. This is because the PCR alone only gave an indication of the presence of the mRNA and not whether they were translated to protein.

3.6.1.1 Source of antibodies used

Due to the lack of commercially available CSE antibodies, Sigma-Genosys custom designed and synthesised an antibody to the CSE enzyme. Using the rat CSE peptide sequence, an antigenic peptide was designed and the sequence chosen was KLH-[C]HASVPEKDRATL. The reason for choosing this sequence is that this peptide sequence is very hydrophilic and located towards the C-terminus of the rat CSE peptide sequence. This will facilitate the binding of the resulting antibody to the CSE protein in the tissue samples tested. The antibodies were raised in two New Zealand White rabbits, using the antibody production schedule outlined below in figure 3.5.

Day	Date	Action	
0	12/06/06	Pre-immune serum collection	5 ml
0	12/06/06	Immunise	200 µg CFA
14	26/06/06	Immunise	100 µg IFA
28	10/07/06	Immunise	100 µg IFA
42	24/07/-6	Immunise	100 µg IFA
49	31/07/06	Bleed	Test bleed #1 ELISA (5 ml)
56	07/08/06	Immunise	100 µg IFA
63	14/08/06	Bleed	Test bleed # 2 (5 ml)
70	21/08/06	Immunise	100 µg IFA
77	28/08/06	Bleed	Test bleed #3

Figure 3.5 CSE antibody production schedule performed over a 77 day incubation period. Test bleed samples 1 and 2 were used for Western blotting against rat ventricle, atria, liver and cerebellum samples to test for specificity. At the end of the 77 day incubation period, the rabbits were scarified and a final bleed (test bleed 3) was performed. The antibody was supplied in rabbit serum and was the most specific of the samples obtained. CFA = Complete Freund's adjuvant, IFA = Incomplete Freund's adjuvant.

During the 77 day incubation program two test bleeds were taken at day 49 and day 63 so that the antibody could be tested for its specificity to detect the CSE protein. ELISA was also performed by Sigma-Genosys to measure the quantity of peptide-specific antibody present in the serum sample from test bleed 1. The final test-bleed was believed to be the most specific antibody to CSE due to the increased incubation period in the rabbit.

In addition to the synthesised CSE antibody, anti-probasin antibodies were also used for the detected of CSE protein. These were a kind gift from Dr Nishi (Department of Endocrinology, Kagawa Medical School, Japan) (Nishi *et al.*, 1992; Nishi *et al.*, 1994). The anti-probasin antibodies had previously detected an unknown 40 kDa protein in rat liver and kidney urinary tubules (Nishi *et al.*, 1992). Investigation of its amino acid sequence (Nishi *et al.*, 1994) revealed it to be almost completely identical with the rat cystathionase reported by Erickson (1990). Probasin and CSE are not homologous, except for the area in which the probasin antibody detected against. Previously these antibodies have successfully been used by Cheng *et al.* (2004) to investigate CSE protein expression in rat mesenteric arteries. Antibodies to the CBS protein were commercially available and a CBS antibody previously tested in rat tissue was purchased from Santa Cruz, USA. Complementary secondary antibodies were purchased from Upstate Cell Signalling Solutions, UK. With the exception of the CSE antibody which was prepared in rabbit serum, the antibodies were prepared in dH₂O.

3.6.1.2 Sample preparation

Initially protein extracts were prepared by homogenising small quantities (5x5 mm) of snap frozen tissue samples in liquid nitrogen and suspending them in 210 µl of lysis buffer containing a protease inhibitor cocktail (Sigma-Aldrich, UK) (see Appendix 3 for composition). Tissue sample from rat left ventricle, right ventricle, liver and brain (cerebellum) were used. The resuspended samples were sonicated for 5 seconds and spun at 13k rpm for 15 minutes. The supernatant was collected and aliquoted. An equal volume of Laemmli sample buffer (see Appendix 3 for

composition) (4% sodium dodecyl sulphate (SDS), 20% glycerol, 10% 2-mercaptoethanol, 0.0004% bromophenol blue, 0.125 M Tris HCl), was added prior to storage at -80°C. A 20 µl aliquot of protein sample prior to the addition of loading dye was kept to be used in the bicinchoninic acid (BCA) protein assay to determine the protein concentrations in the tissue samples (see Chapter 2, section 2.3).

3.6.1.3 Separation of proteins

The protein samples were heated to 95 °C for 5 minutes. Equal protein concentrations were loaded onto a 4% stacking gel (see Appendix 3 for composition), with a maximum volume of 20 µl being loaded to achieve the desired concentration. The specific protein concentrations loaded for each antibody are shown in figure 3.6. 5 µl of Bio-Rad precision plus protein standard (Bio-Rad Laboratories Inc., UK) was also loaded. The proteins were separated on a 10 % resolving gel (see Appendix 3 for composition) for 1 hour at 150 V using a mini-PROTEAN 3® cell and tank system (Bio-Rad Laboratories Inc., UK).

3.6.1.4 Preparation of PVDF membrane and probing for CSE protein

Electrophoretic transfer was used to transfer the separated proteins to a polyvinylidene difluoride (PVDF) membrane (Hybond P, Amersham Biosciences Ltd., UK). The PVDF membrane was initially activated by soaking in methanol for 1 minute and then washing in dH₂O. The protein transfer took 1 hour and was performed using the mini Trans-Blot module® (Bio-Rad Laboratories Inc., UK) with the power pack set to 100 V and 400 mA. After the protein transfer was complete the membrane was with blocked in a 5% (w/v) milk solution (5 ml), made up in Tris-buffered saline TBST (see Appendix 3 for composition), with constant agitation on a roller shaker for 60 minutes. The membrane was then placed in a 5% (w/v) milk solution containing a 1:1000 dilution of primary antibody.

The generalised probing method is as follows. The specific antibody concentrations and blocking conditions for each protein of interest are summarised in figure 3.6. The blots were incubated with the primary antibody at 4 °C overnight on a roller

shaker. The following morning 3, 5 minute washes of the membrane were performed in 10 ml of TBST buffer solution. The membrane was then incubated in a 5% (w/v) milk solution containing secondary antibody for 90 minutes. The secondary antibodies were raised in a different species to the primary antibody, and cross reacted. The membrane received a further 3, 5 minute washes in TBST. The bound antibodies were detected by the secondary antibody conjugated to horseradish peroxidase and visualised by chemiluminescence (ECL plus™ Western Blotting Detection Reagents, Amersham Biosciences Ltd., UK). The bands were captured on photographic film (Hyperfilm™ ECL, Amersham Biosciences Ltd., UK). After development and fixation on the films, they were scanned on an EPSON perfection 2480 photocopier (EPSON Ltd., UK).

Antibody	[optimum protein] (µg/µl)	[1°]	[2°]	Blocking agent
CSE	100	1:500	1:20k	5% milk
CBS	100	1:500	1:20k	5% milk
Probasin-monoclonal	37	1:1000	1:10k	5% milk
Probasin-polyclonal	37	1:1000	1:10k	5% milk

Figure 3.6 Summary of the optimum Western blotting conditions for each of the antibodies used to characterise CSE and CBS enzyme expression in the rat myocardium, liver and cerebellum.

After developing and scanning the blots, they were stripped of the primary and secondary antibodies by washing in 5 ml stripping buffer (Restore™, Pierce, UK) for 30 minutes on a roller shaker. The blots were washed briefly in 10 ml TBST and then received 2, 10 minute washes in 20 ml TBST. The blots were then re-probed with an antibody to beta actin (1:5000) in 5% (w/v) milk overnight. The blots received 3, 5 minute washes in TBST and were then incubated with secondary antibody (1:5000) in 5% milk for 90 minutes. The bound antibodies were detected by the secondary antibody conjugated to horseradish peroxidase and visualised by chemiluminescence as described previously. This method was also used for the stripping and re-probing

the CSE and CBS antibody probed blots, except that they were re-probed with either the CSE or CBS antibody in an attempt to reduce to reduce the excess protein that results in non-specific binding reducing the clarity of the bands of interest.

The effect of blocking the membranes in 5% BSA was also investigated to see if it was more effective than milk. However, the resulting blots had far more non-specific binding and so 5% (w/v) milk was used as a blocking agent in all subsequent experiments.

3.7 Use of a HiTrap™ protein G HP purification column for purification of CSE antibody

A 1ml HiTrap™ protein G HP purification column (GE Healthcare LTD, UK) was washed with 15 volumes of binding buffer (15ml total) (see Appendix 3 for composition) at a rate of 1 ml/min. 1ml of rabbit final bleed was applied to the column. The column was left plugged at room temperature for 1 hour. The column was washed with 15 volumes of binding buffer (15ml total) before the antibody was eluted with 8 column washes of elution buffer (8ml total) (see Appendix 3 for composition). Each of the 1 ml eluted fractions was collected into an eppendorf tube containing 50µl Tris-HCl (1 M, pH 9.0). The protein concentrations of the eluted fractions were measured on a Labtech ND 1000 spectrophotometer (Fisher Thermo Scientific, UK) connected to a computer running analysis software version 3.2.1. The second fraction was found to contain the greatest amount of protein and was used as the primary antibody sample for all subsequent CSE western blotting, at a dilution of 1:500.

3.8 Cloning and over-expression of the CSE enzyme

Cloning and over-expression of the CSE enzyme in a GH3 cell line was performed by Dr. Rob Fowkes and Dr. Steve Allen (RVC, London), as outlined in Appendix 1. Whilst I did not perform the experimental work myself I was involved in the planning and

concepts behind the studies. I did however perform the Western blot analysis of the transfected cell line over-expressing the CSE enzyme.

3.9 Results

3.9.1 PCR studies for CSE and CBS mRNA expression

3.9.1.1 CSE mRNA expression in rat tissue samples:

The CSE mRNA expression levels were investigated in rat tissue samples using PCR. The aorta, left ventricle and right ventricle tissue homogenates from rats all showed weak expression of CSE mRNA. Liver and brain homogenates contained stronger expression levels (as shown in figure 3.7). It is not possible to quantify the expression levels because unequal amounts of cDNA were used in each sample.

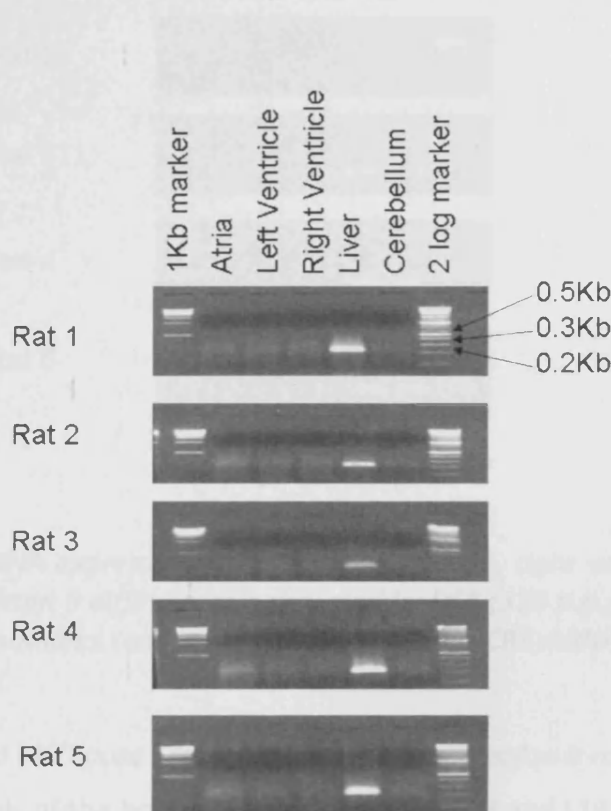


Figure 3.7 CSE mRNA expression in aorta, left ventricle, right ventricle, liver, and brain (cerebellum) from 5 different rats was detected by PCR (234 b.p., 35 cycles). Bands appear in all tissue samples confirming the expression of CSE mRNA.

3.9.1.2 CBS mRNA expression in rat tissue samples

The CBS mRNA expression levels were investigated in rat tissue samples using PCR. The aorta, left ventricle, right ventricle, liver, and cerebellum tissue homogenates

from rats all contained detectable mRNA for CBS. The liver and cerebellum appeared to contain stronger expression levels (as shown in figure 3.8).

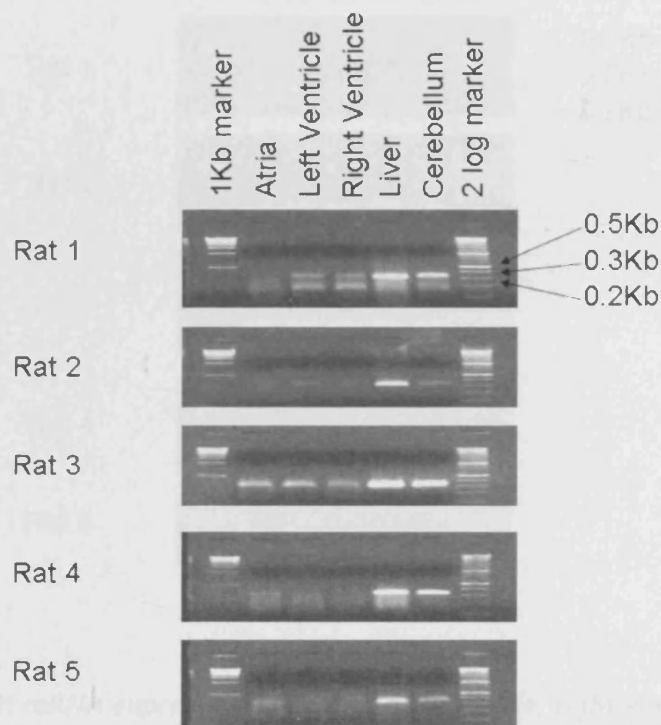


Figure 3.8 CBS mRNA expression in aorta, left ventricle, right ventricle, liver, and brain (cerebellum) from 5 different rats detected by PCR (325 b.p., 35 cycles). Bands appear in all tissue samples confirming the expression of CBS mRNA.

3.9.1.3 GAPDH and L19 house keeping gene mRNA expression in rat tissue samples

The expression levels of the house keeping genes GAPDH and L19 were investigated in rat tissue samples using PCR. All of the samples contained strong expression levels of mRNA for the house keeping genes GAPDH (as shown in figure 3.9) and L19 (as shown in figure 3.10), except in the cerebellum sample from rat 5 where surprisingly no GAPDH expression was detected.

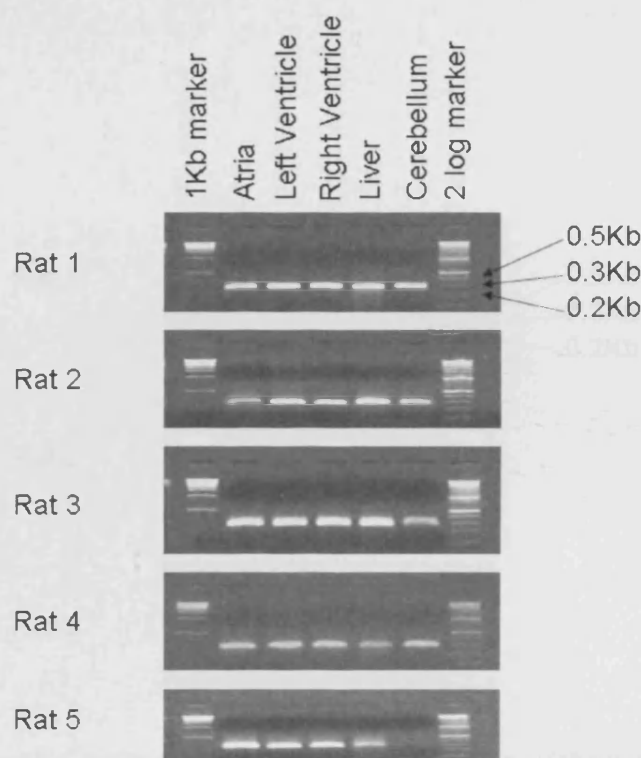


Figure 3.9 Gel electrophoresis image showing GAPDH mRNA expression in aorta, left ventricle, right ventricle, liver, and brain (cerebellum) from 5 different rats detected by PCR (294 b.p., 35 cycles). Bands appear in all tissue samples confirming the expression of GAPDH mRNA.

Figure 3.9 GAPDH mRNA expression in aorta, left ventricle, right ventricle, liver, and brain (cerebellum) from 5 different rats detected by PCR (294 b.p., 35 cycles). Bands appear in all tissue samples confirming the expression of GAPDH mRNA.

3.9.2.1 Western blotting using the CSE antibody from rat liver (Fig. 3.10)

Western blotting was used to characterize the expression of the CSE enzyme in the rat myocardium, cerebellum and liver. The initial Western blotting was performed with the anti-CSE 1 serum containing the CSE antibody and showed bands present around the position of 40 kDa which is the weight of the CSE protein (data not shown). The bands were most prevalent in liver and cerebellum samples. The left and right ventricles contained fainter bands. The blots also showed a large amount of non-specific binding and a lot of background noise in the Western blotting. To improve the quality of the blots (Fig. 3.10), the method of Western blotting was improved.

3.9.2.2 Western blotting using the housekeeping protein beta actin

Antibodies against the house-keeping protein beta actin were used to reprobe the blots after they had been stripped of the CSE antibodies. The blots showed

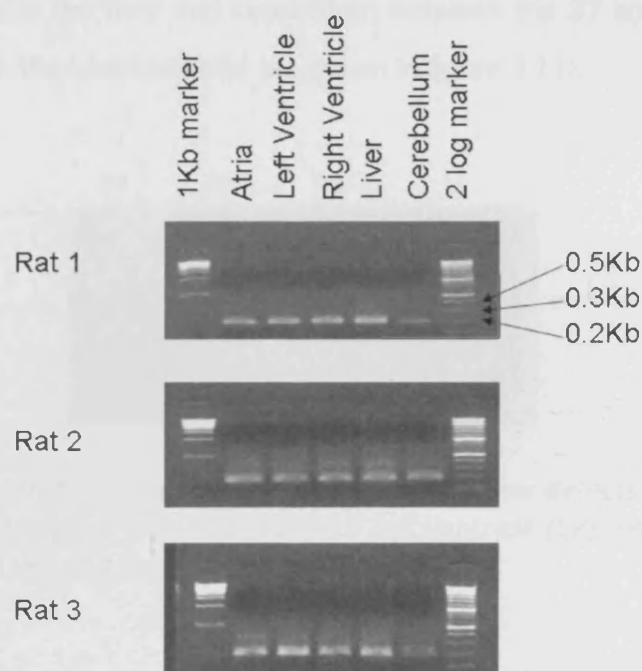


Figure 3.10 *L19 mRNA expression in aorta, left ventricle, right ventricle, liver, and brain (cerebellum) from 3 different rats detected by PCR (194 b.p., 35 cycles). Bands appear in all tissue samples confirming the expression of L19 mRNA.*

3.9.2 Western blotting for CSE protein expression

3.9.2.1 Western blotting using the CSE antibody from test bleed 1

Western blotting was used to characterise the expression of the CSE enzyme in the rat myocardium, cerebellum and liver. The initial Western blotting was performed with the test bleed 1 serum, containing the CSE antibody, and showed bands present around the position of 40 kDa which is the weight of the CSE protein (data not shown). The bands were most prevalent in liver and cerebellum samples. The left and right ventricles contained fainter bands. The blots also showed a large amount of non-specific binding and refinements were made to the Western blotting to improve the quality of the blots (as outlined in the methods section).

3.9.2.2 Western blotting using the housekeeping protein beta actin

Antibodies against the house keeping protein beta actin were used to reprobe the initial blots after they had been stripped of the CSE antibodies. The blots showed

very strong bands in the liver and cerebellum between the 37 and 50 kD markers, but were fainter in the heart samples (as shown in figure 3.11).

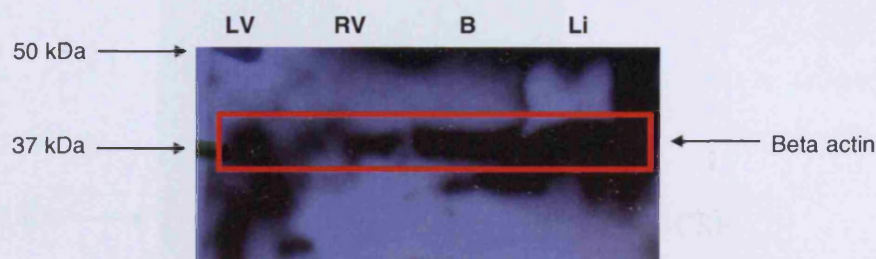


Figure 3.11 Western blot using a beta actin antibody that detects the protein at 40 kD. Protein samples were obtained from rat left ventricle (LV), right ventricle (RV), brain (cerebellum) (B), and liver (Li).

3.9.2.3 Western blotting using the CSE antibody from test bleed 3

After refinement of the Western blotting technique, the study was repeated using the CSE antibody from the final test bleed (bleed 3) to test for the expression of the CSE enzyme protein. The blot shows two sets of bands appearing in all of the protein samples between the 35 and 50 kD markers suggesting CSE protein may be present in the left ventricle, right ventricle, cerebellum and liver tissue samples from the rat (as shown in figure 3.12). However, due to multiple bands appearing within close proximity of each it is difficult to distinguish which band is at the correct molecular weight, 40 kDa, for CSE. The blots also appear to still contain a large amount of non-specific binding and noise, but are a vast improvement on the initial blots obtained with test bleed 1 and 2. Stripping and re-probing with the same primary antibody also produced a marked improvement in clarity of the bands of interest, compared with un-stripped blots.

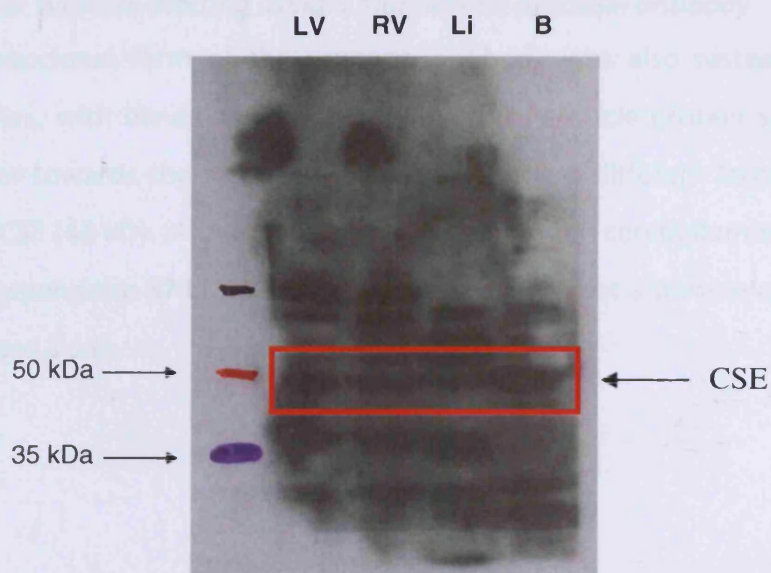


Figure 3.12 Western blot using a custom CSE antibody, raised in the rabbit by Sigma Genosys, that detects the CSE enzyme at 40 kD. Protein samples were obtained from rat left ventricle (LV), right ventricle (RV), and liver (Li), and brain (cerebellum) (B).

3.9.2.4 Western blotting using a polyclonal probasin antibody

The results from Western blotting performed using a polyclonal probasin antibody (IgG fraction) from Dr Nishi showed bands appearing in right ventricle, cerebellum and liver protein samples between the 37 and 50 kD markers suggesting the CSE enzyme protein is present (as shown in figure 3.13).

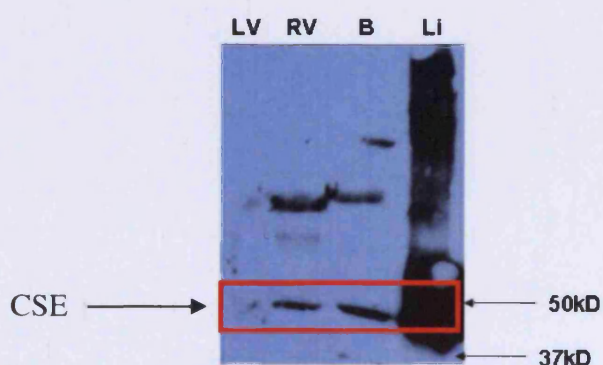


Figure 3.13 Western blot using a polyclonal probasin antibody that detects the CSE enzyme at 40 kD. Protein samples were obtained from rat left ventricle (LV), right ventricle (RV), brain (cerebellum) (B), and liver (Li). 30 μ g of protein was loaded in each lane.

3.9.2.5 Western blotting using a monoclonal probasin antibody

A monoclonal form of the probasin antibody was also tested in the rat protein samples, with bands appearing only in right ventricle protein sample. These were further towards the top of the blot, suggesting a different larger product detected than CSE (43 kD). A band was also detected in the cerebellum sample lane, but this was beyond the 37 kD marker and was therefore not a protein of interest (as shown in figure 3.14).

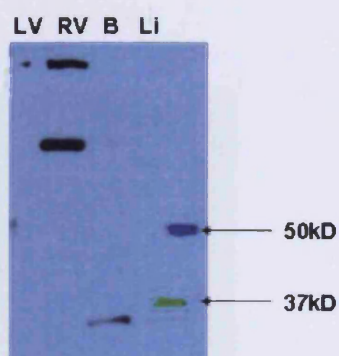


Figure 3.14 A Western blot using a monoclonal probasin antibody, that can detect the CSE enzyme at 40 kD. Protein samples were obtained from rat left ventricle (LV), right ventricle (RV), brain (cerebellum) (B), and liver (Li). 30 μ g of protein was loaded in each lane.

3.9.2.6 Western blotting using the CBS antibody

When probing with the CBS antibody only a band was detected in the liver sample, but this was not at the expected size of 63 kDa and appears to be closer to 40 kDa (as shown in figure 3.15). There appears to be no CBS protein in the myocardium and cerebellum samples. Stripping and re-probing the blots with the CBS antibody showed a marked improvement in clarity of the bands if interest, compared with unstripped blots but still did not produce bands in the other sample lanes.

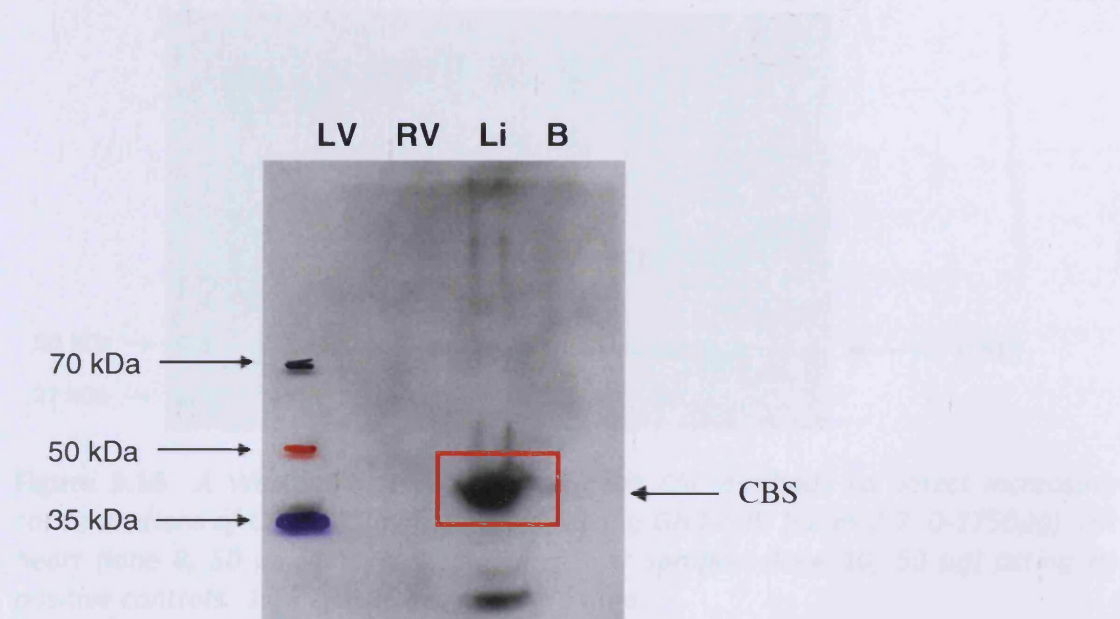


Figure 3.15 Western blot using an CBS antibody to detect the CBS enzyme at 63 kD. Protein samples were obtained from rat left ventricle (LV), right ventricle (RV), liver (Li), and brain (cerebellum) (B).

3.9.2.7 Western blotting using the CSE over-expressing GH3 cells and CSE antibody

There is a band that appears at approximately 40 kDa across all the GH3 cells over-expressing CSE protein which appeared to increase in expression coincident with an increase in the amount of CSE expression vector transfected (as shown in figure 3.16). Bands also appeared at the same position in the heart and liver samples which acted as positive controls.

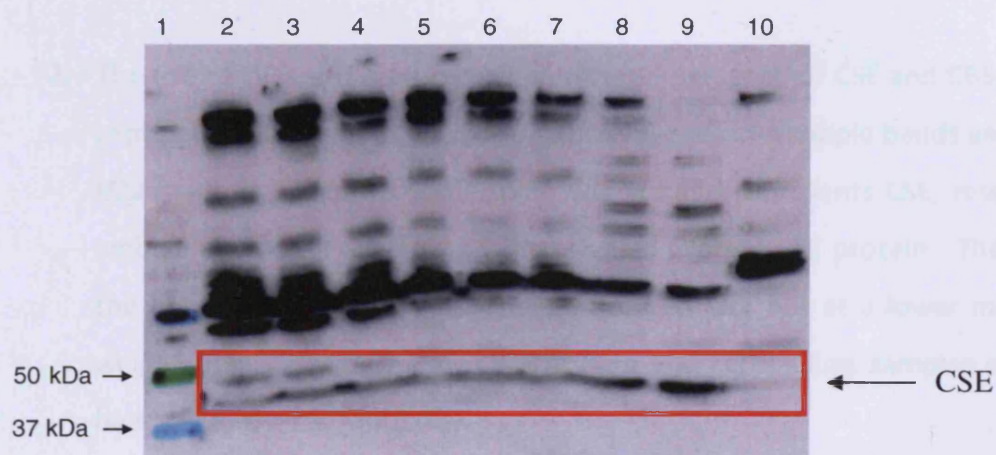


Figure 3.16 A Western blot probed using the CSE antibody to detect increasing concentrations of CSE protein in over-expressing GH3 cells (lanes 2-7, 0-1750 μ g), rat heart (lane 8, 50 μ g, lane 9, 70 μ g) and liver samples (lane 10, 50 μ g) acting as positive controls. 1:250 1° , 10 sec exposure time.

3.10 Discussion

The findings of this study can be summarised briefly as follows:

1. PCR has shown that mRNA for both the CSE and CBS enzymes is present in the aorta, left ventricle, right ventricle, liver and cerebellum samples from rat. Relatively stronger CSE mRNA expression was observed in the liver, whilst the cerebellum and liver showed stronger bands for CBS mRNA.
2. The use of Western blotting to confirm the presence of CSE and CBS protein expression was of limited success. The presence of multiple bands around 40 kDa made it difficult to determine which band represents CSE, resulting in ambiguity when interpreting which tissues express CSE protein. The liver is the only sample to show a band on the CBS blot but at a lower molecular weight than expected. The myocardium and cerebellum samples show no bands with the CBS antibody.
3. Cloning, transfection, and subsequent over-expression of the CSE protein in a GH3 cell line was an attempt to decipher which of the bands observed with the CSE antibody was the correct molecular weight for CSE. However, this work was inconclusive, providing further evidence that the CSE antibody exhibited strong non-specific binding.

3.10.1 PCR

3.10.1.1 CSE mRNA is present in all tissue samples

The PCR results using the CSE primers suggest that CSE mRNA expression exists in rat aorta, left ventricle and right ventricle. There appears to be stronger CSE mRNA expression in the rat liver, although this was not a quantitative technique. The liver acted as a positive control for the expression of CSE mRNA. It has been previously reported in that CSE mRNA is predominantly expressed in myocardial tissues (Abe *et al.*, 1996). However, there appears not to have been any previous studies that have divided the heart into specific regions, as in this series of experiments. Also the definition of myocardial tissue appears vague and may in fact refer to the aorta and

other regions of the cardiovascular system rather than specific major components of the heart such as the atria and ventricles. This makes a comparison of results with the literature difficult. CSE mRNA expression was also reported to be undetectable in the brain (Abe *et al.*, 1996), but results from this study suggest that weak expression levels of CBS mRNA expression were present.

3.10.1.2 CBS mRNA is also present in all tissue samples

The results from the PCR using the CBS primers, suggest that the CBS mRNA is expressed in all of the rat tissue samples tested. There appears to be stronger expression in the liver and the cerebellum, although again this was not a quantitative technique. This supports previous findings that the liver and cerebellum strongly express the CBS enzyme (Abe *et al.*, 1996; Hosoki *et al.*, 1997). The expression of CBS protein in the rat myocardium requires confirmation using Western blotting. Previously, no expression of CBS protein in human atrium and ventricle tissues has been shown (Chen *et al.*, 1999). It would be interesting to see if there is a species difference in the CBS protein expression between human and rats.

It is not surprising that the liver contained strong expression of CSE and CBS mRNA, since it has been reported that the liver strongly expresses these enzymes (Hosoki *et al.*, 1997); (Wang, 2002). Another explanation for the strong expression of CSE and CBS mRNA in the liver is that a better preparation of mRNA may have been obtained, since it does not contain as much collagen and therefore it may be easier to extract the RNA than the myocardial tissues. As a result this may have improved the cDNA synthesis and PCR reactions, producing brighter bands on the gel. The mRNA expression of the house keeping genes GAPDH and L19 are positive in all but one tissue sample (rat 5 cerebellum sample used to detect GAPDH), suggesting that the integrity of the cDNA was sufficient for the PCR reactions to be successful. It also appears that there is expression is consistent both within and between tissue types.

It has been proposed in the literature that there may be an age dependent expression of CSE in cardiovascular tissue (Levonen *et al.*, 2000). This idea was

proposed based on the observation that in fetal, premature, and full term neonatal liver tissue there is an absence of CSE activity, compared with the adult liver (Levonen *et al.*, 2000). This suggests that there is an age dependent posttranscriptional regulation of the CSE enzyme. After 5 weeks the activity of the CSE enzyme has reached levels comparable to adults (Kuo *et al.*, 1983). Since the liver tissue samples from all of the rats, showed strong CSE mRNA expression, it suggests that if CSE activity was age related and that the rats used in this study (7-8 weeks old) were mature enough to express the CSE enzyme. Therefore the weak expression in the myocardium was not as a result of their young age. The age dependent expression of CSE present in cardiovascular tissues has not previously been studied (Wang, 2003).

3.10.1.3 Limitations associated with PCR

The main limitations associated with the PCR technique used to obtain the data presented in this chapter are: poor precision, low sensitivity, short dynamic range, low resolution, and the data obtained are not quantitative. It is also a relatively labour intensive technique due to being non-automated and requires post PCR processing of the products. Perhaps the major limiting factor though is that data is obtained from a single point at the end of the amplification process. This means that is if all the samples have replicated from differing amounts of DNA, but have all reached the plateau phase by the end of the series of cycles, then the measurement taken at the plateau phase is not representative of the initial amounts of starting material. It would be far more accurate to take measurements during the exponential phase when the samples are amplifying exponentially.

3.10.1.4 Quantitative PCR techniques

Real time PCR (RT-PCR) enables quantitative determination of total RNA expression. RT-PCR collects data in the exponential growth phase of the experiment and measures the amount of total RNA at time points throughout the duration of the experiment. This technique relies on a reporter fluorescent signal that increases proportionally to the number of amplicons generated. It is a more accurate

technique for DNA and RNA quantification and doesn't require laborious post PCR processing.

3.10.2 Western blotting

3.10.2.1 Initial blots with the CSE antibody suggests impurities in the serum

Preliminary results from Western blotting using the CSE antibodies suggest that the rabbit serum containing the CSE antibody was not that pure and there may be other factors present resulting in non-specific binding of the antibody. Attempts were made to improve the clarity of the blots by reducing the primary antibody concentration, increasing the concentration and duration of milk blocking, reducing the amount of protein loaded, and stripping and re-probing the blots to reduce non-specific binding. These measures appear to have significantly improved the clarity of the blots, but they are still far from ideal. Purification of the antibody by running the sample through an IgG column was also performed in an attempt to reduce the possibility of non-specific binding from other serum factors. However, despite these refinements to the Western blotting techniques, there still appears to be too much non-specific binding which creates ambiguity as to which bands represent CSE.

The blot in figure 3.12 shows two series of bands between the 35 and 50 kDa marker. The series of bands that appear at 45 kDa in all of the tissue samples suggest the presence of CSE protein. There is also an additional band in the liver sample lane at a slightly higher molecular weight with a greater band intensity. This implies that the liver could be expressing a splice variant with a heavier molecular weight. It is possible that the CSE protein in the liver sample is more abundantly expressed due to being more active. As a result increased activation may result in increased phosphorylation and an increase in molecular weight. Running a BLAST search with the peptide sequence for CSE, revealed that there are two possible phosphorylation sites, as well as two PKC-target sites. These may account for a slight change in molecular weight of the CSE protein. However, CSE is unlikely to be heavily phosphorylated due to the reducing conditions used for the protein separation. The series of bands that occur just above the 35 kDa marker are less

likely to be CSE because the liver band is very weak, which seems uncharacteristic of its strong mRNA expression.

The CSE protein expression in the rat myocardium was unconfirmed using Western blotting due to the lack of specificity of the antibody. An attempt to overcome the poor specificity of antibody was performed by preparing transcripts containing increasing amounts of a CSE expression vector that were inserted into the GH3 cell line, to create cells with corresponding increases in CSE protein expression. When these cells were lysed and Western blotting was performed with the CSE antibody, there was an expected increase in the band density across the blot as the amount of CSE expression vector and subsequent CSE protein increased. The idea behind this method was that the position of the bands of increasing density represented the CSE protein, allowing it to be distinguished from the multiple series of bands appearing on the blots. Nevertheless, due to the considerable extent of the non-specific binding observed in the transfected GH3 extracts, it is difficult to determine whether the band at 40 kDa truly represented the immunoreactive CSE protein. What these findings do confirm is that the antibody to CSE was highly unspecific, making the antibody of limited use for protein determination using Western blotting.

3.10.2.2 Lack of experimental evidence for CSE expression in the myocardium

It has been reported that the expression of CSE in the myocardium is unknown (Geng *et al.*, 2004c), and the lack of a commercially available CSE antibody (Zhao *et al.*, 2001a) has prevented the robust detection of the CSE protein. Studies conducted by Ishii *et al.* (2004) who produced their own CSE antibody, gave equivocal evidence of CSE protein expression in the myocardium. A recent study by Chuah *et al.* (2007) has confirmed the presence of CSE protein in the left ventricle from rats, although no details of the source of antibodies is given. Therefore the evidence supporting statements in the literature by authors such as Fiorucci *et al.* (2005) and Geng *et al.* (2004c) that CSE is the predominant enzyme in the myocardium appears somewhat unfounded. Clearly there is a great need for a commercially available CSE antibody to

improve the quality of Western blots and enable confident characterisation the CSE protein expression.

3.10.2.3 Polyclonal anti-probasin antibody detects CSE in right ventricle and liver

The anti-probasin mono and polyclonal antibodies were a generous gift from Dr. Nishi in Japan (Nishi *et al.*, 1992). The blots presented in this chapter using the polyclonal antibodies suggest expression of the CSE protein in the right ventricle, cerebellum and liver. Surprisingly there appears to be an absence of a band in the left ventricle lane, suggesting the absence of the CSE enzyme. There appears to be no reason as to why the right ventricle would express the protein and the left would not. Previously reported data from Dr. Nishi's laboratory using the polyclonal antibody show has shown bands at 43 kDa in aorta, mesenteric artery, tail artery smooth muscle, and pulmonary artery from rat tissue samples (Cheng *et al.*, 2004). The results using the monoclonal form of the antibody were less encouraging with no bands of the correct size in any of the tissue samples tested. There were only bands observed in right ventricle and cerebellum column much further up the blot suggesting the presence of heavier proteins. It was surprising that even the liver sample didn't show a band since it appears to be a robust tissue sample that has consistently shown expression with the other antibodies tested. These findings suggest that either the antibody was either not that specific to the CSE protein making it unable to detect it, or it had become denatured making it ineffective at detecting the CSE protein.

3.10.2.4 CBS antibody has poor selectivity for CBS

The liver was the only tissue sample to show a band when using the CBS antibody. The position of the band was also at approximately 45 kDa as opposed to the expected molecular weight of 63 kDa. However, it is known that a dimer of CBS exists with a molecular weight of 48 kDa (Skovby *et al.*, 1984). It this thought that CBS is originally formed into a tetramer structure of 63 kDa and that the dimer form occurs as a result of a specific protease activity. The 48 kDa dimer form is thought to be the catalytically active form of the 64 kDa tetramer. In fact previous

attempts to purify CBS protein from mammalian livers have yielded enzyme containing subunits with a molecular weight lower than 63 kDa (Borcsok *et al.*, 1982; Kraus *et al.*, 1978). However, the existence of a dimer form of CBS is unlikely to be detected using the Western blotting techniques outlined in this chapter, due to the reducing conditions when separating the proteins. Only if the reducing conditions were not entirely efficient, would enable protein-protein interactions remain.

There appears to be no CBS protein in the myocardium and cerebellum samples. Whilst it has been previously reported that CBS expression is weak/absence in the myocardium (Zhao *et al.*, 2002), the lack of a band in the cerebellum sample which is thought to predominantly express the CBS express was an unexpected finding. These findings suggest that the Santa Cruz antibody used to detect CBS perhaps had poor selectivity for CBS. Ideally a CBS antibody from a different company should have been tested and the results compared. However, at the time of experimentation Santa Cruz were the only suppliers of a CBS antibody that had been tested in rat and was therefore the primary choice.

Another possible explanation for the lack of bands in the cerebellum and myocardium lanes is that the liver contained such a high abundance of CBS that it masked the weaker expression in the other tissues in the short exposure times of the blots to the photographic film. This could have been investigated by exposing the blots to the photographic film for longer to see if any bands appeared in the lanes of the cerebellum and myocardium samples. However, previous experience of over-exposed films usually resulted in increased background noise making it very difficult to determine the position of the bands on interest.

3.10.2.5 Further uses for antibodies

The antibodies used in the Western blotting could also be used for immunohistochemistry to enable localisation of the H₂S producing enzymes in the myocardium. Sections from different regions of the heart could be prepared and the presence of the enzymes investigated to see if there is regional variation in

distribution. Some preliminary work has been conducted using transverse sections of heart tissue. Unfortunately, the unspecific nature of the CSE antibody resulted in a large amount of background noise appearing on the slides, making it difficult to conclude with confidence the areas that were actually staining positive for CSE. Immunohistochemistry with the CBS antibody showed no staining in any of the regions of rat myocardial tissue samples. Taken in isolation these results suggest that the rat myocardium lacks the CBS enzyme. However, taking these findings in the context of the Western blotting studies showing a lack bands in the positive control cerebellum sample, suggest that these findings are of limited value and can't be confidently accepted. Had time permitted it would have been interesting to repeat these studies and also use slices of cerebellum and liver tissue to act as positive controls to give greater confidence in the observed results.

3.11 Conclusion

The poor performance of the antibodies, in particular CSE, used in the Western blotting studies makes it difficult to draw a definitive conclusion about the expression of the H₂S synthesising enzymes in the myocardium from the studies in this chapter. This issue of poor selectivity was addressed during the course of the studies and a conscious effort was made to try and characterise the CSE antibody further through the use of an expression vector for CSE in a GH3 cell line. However, this too was of limited success, but did act to further highlight the issue surrounding the poor selectivity of the CSE antibody, as opposed to the Western blotting technique. Therefore it can be concluded that the CSE antibody appears highly unspecific to the CSE protein and also contains contaminants in the serum which effect the quality of the blots. However, the PCR data suggests that the rat myocardium, liver and cerebellum all contain CSE and CBS mRNA expression, which suggests these tissue have the potential to express CSE and CBS protein enabling H₂S production. Further evidence to support the existence of H₂S synthesising enzymes is provided in studies investigating the production of H₂S gas after endogenous stimulation with substrate and co-factor, which are presented in chapter 4 and 5.



Chapter 4 Exogenous stimulation of H₂S production using L-cysteine to limit infarct size during ischaemia-reperfusion

4.1 Introduction:

An extensive overview of the H₂S synthesis pathway has been reviewed in Chapter 1. Studies performed in the previous chapter (chapter 3) attempted to explore the expression of the H₂S synthesising enzymes (CSE and CBS) in the rat myocardium at both the mRNA and expressed protein level. Whilst the results from the PCR studies confirm the potential for protein expression from positive mRNA findings, there was no definitive proof from Western blotting studies of protein expression. The studies in this chapter aimed to build on these previous findings and further characterise the existence of the CSE/CBS enzymes in the rat myocardium, by exogenously stimulating H₂S production and observing the effect on infarct size limitation. As mentioned previously in Chapter 1, endogenous H₂S synthesis predominantly occurs via the CSE and CBS enzymes that use L-cysteine as substrate (Szabo, 2007) and pyridoxal-5'-phosphate (P-5'-P) as co-factor (Stipanuk *et al.*, 1982). Therefore in the studies conducted in this chapter and in Chapter 5, endogenous H₂S synthesising conditions were mimicked. A specific pharmacological inhibitor to CSE, DL-propagylglycine (PAG), was also used to confirm the involvement of the CSE enzyme, since this is thought to be the key enzyme responsible for H₂S production in the myocardium (Abe *et al.*, 1996; Geng *et al.*, 2004b).

The table in figure 4.1 summarises a number of published PCR studies characterising the H₂S synthesising enzymes in various tissue samples including the myocardium, providing further evidence to support the potential to exogenously stimulate H₂S in the myocardium.

Species	Tissue	Reference
Rat	Heart, brain, thoracic aorta	(Geng <i>et al.</i> , 2004c)
Rat	Thoracic aorta	(Zhong <i>et al.</i> , 2003)
Rat	Left ventricle, right ventricle	(Zhu <i>et al.</i> , 2007)
Rat	Aortic vascular smooth muscle cells from	(Zhong <i>et al.</i> , 2009)
Rat	Aorta	(Wu <i>et al.</i> , 2006)
Rat	Endothelium-free pulmonary artery, mesenteric artery, tail artery, aorta, liver	(Zhao <i>et al.</i> , 2001a)
Human	Aortic smooth muscle cells	(Yang <i>et al.</i> , 2006)

Figure 4.1 Summary of published PCR studies characterising the CSE enzymes in various tissues including the myocardium.

4.2 Aim

The aim of this series of experiments was to investigate if exogenous stimulation of the H₂S synthesising enzyme CSE, with its substrate L-cysteine and co-factor P-5'-P, limits infarct size in Langendorff perfused rat hearts subjected to regional ischaemia-reperfusion.

4.3 Hypotheses

1. Stimulated H₂S production using L-cysteine, will limit infarct size in rat hearts subjected to regional ischaemia-reperfusion
2. Addition of the CSE co-factor P-5'-P will enhance H₂S production and further limit infarct size in rat hearts subjected to regional ischaemia-reperfusion

4.4 Objectives

The validity of these hypotheses was tested as follows:

1. To determine of the optimum concentration of L-cysteine to induce cardioprotection
2. To determine if cardioprotection is induced by L-cysteine stimulated H₂S production using the CSE specific inhibitor PAG

4.5 Methods

4.5.1 Source of compounds

The CSE substrate L-cysteine, the CSE inhibitor DL-propagylglycine (PAG) and the CSE co-factor P-5'-P (P-5'-P) were purchased from Sigma-Aldrich, UK. All compounds were dissolved in Krebs Buffer and therefore required no vehicle controls.

4.5.2 Preparation of Langendorff perfused isolated rat hearts

4.5.2.1 Setup of isolated heart perfusion

Heart samples used for the maximal H₂S synthesising capacity assay and endogenous H₂S tissue concentration experiments, were prepared from rat isolated perfused hearts.

Isolated rat hearts were Langendorff perfused using the methods described in Chapter 2, section 2.2. All hearts received an initial 10 minute stabilisation period after initially being cannulated to enable a regular sinus rhythm to be obtained. The hearts were then randomly assigned to one of the groups within each study described below. Throughout the experiments the cardiodynamic parameters were measured (heart rate, developed pressure, and coronary flow rate) to ensure that the hearts were maintaining function and to observe any drug induced effects on the normal functioning of the hearts.

4.5.2.2 Treatment protocols

Study 1: Optimum L-cysteine concentration for infarct size limitation

Group 1 - Control: After 20 minutes of stabilisation, hearts were subjected to 35 minutes regional ischaemia, and a 120 minute reperfusion period. For the duration of the experiment hearts were perfused with Krebs buffer.

Group 2 - L-cysteine 0.1 mM: After 10 minutes of stabilisation, hearts were perfused with Krebs containing L-cysteine 0.1 mM for the remaining 10 minutes of stabilisation until the first 10 minutes of reperfusion.

Group 3 - L-cysteine 1 mM: As per group 2 except hearts were perfused with L-cysteine 1 mM.

Group 4 - L-cysteine 10 mM: As per group 2 except hearts were perfused with L-cysteine 10 mM.

The greatest reduction in infarct size was seen with L-cysteine 1 mM, so this concentration was used for the subsequent studies.

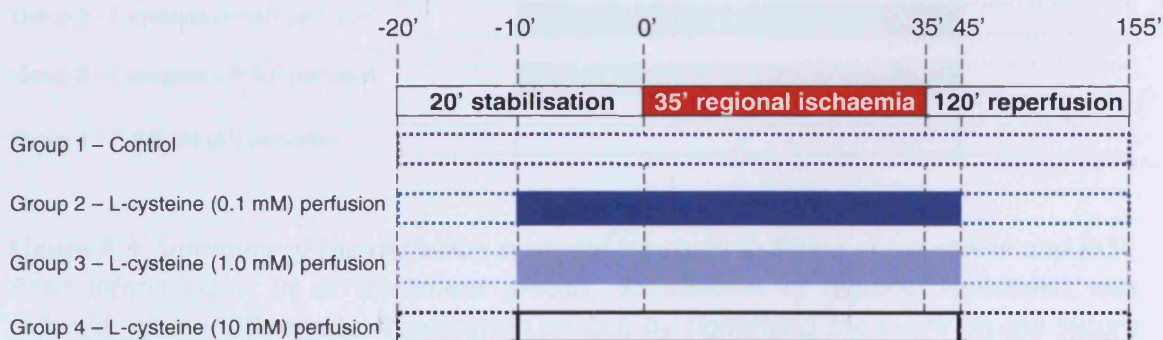


Figure 4.2 Summary of the perfusion protocol for study 1: Optimum L-cysteine concentration for infarct size limitation. In all treatment groups, 35 minutes of regional ischaemia was induced, after a 20 minute stabilisation period, by tightening the snare on the suture passed around the left main coronary artery. Each heart received 120 min reperfusion following ischaemia. After 10 minutes of reperfusion, the treatment hearts were perfused with Krebs for the duration of the perfusion period.

Study 2: Effect of L-cysteine and P-5'-P on infarct size

Group 1 - Control: After 20 minutes of stabilisation, hearts were subjected to 35 minutes regional ischaemia, and a 120 minute reperfusion period. For the duration of the experiment hearts were perfused with Krebs buffer.

Group 2 - L-cysteine 1 mM: After 10 minutes of stabilisation, hearts were perfused with Krebs containing L-cysteine 1 mM for the remaining 10 minutes of stabilisation until the first 10 minutes of reperfusion.

Group 3 – P-5'-P 50 µM: As per group 2 except hearts were perfused with P-5'-P 50 µM.

Group 4 – L-cysteine 1 mM and P-5'-P: As per group 2 except hearts were perfused with L-cysteine 1 mM and P-5'-P 50 µM.

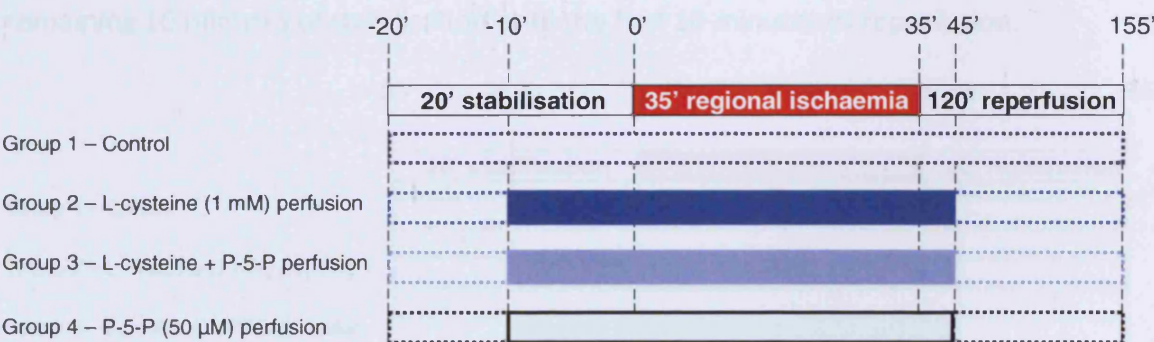


Figure 4.3 Summary of the perfusion protocol for study 2: Effect of L-cysteine and P-5'-P on infarct size. In all treatment groups, 35 minutes of regional ischaemia was induced, after a 20 minute stabilisation period, by tightening the snare on the suture passed around the left main coronary artery. Each heart received 120 min reperfusion following ischaemia. After 10 minutes of reperfusion, the treatment hearts were perfused with Krebs for the duration of the perfusion period.

Study 3: Effect of PAG on infarct size limitation by L-cysteine

Group 1 – Control: After 20 minutes of stabilisation, hearts were subjected to 35 minutes regional ischaemia, and a 120 minute reperfusion period. For the duration of the experiment hearts were perfused with Krebs buffer.

Group 2 - L-cysteine 1 mM: After 10 minutes of stabilisation, hearts were perfused with Krebs containing L-cysteine 1 mM for the remaining 10 minutes of stabilisation until the first 10 minutes of reperfusion.

Group 3 – PAG 1mM: After 5 minutes of stabilisation, hearts were perfused with Krebs containing PAG 1mM until the first 10 minutes of reperfusion.

Group 4 – L-cysteine 1mM + PAG 1 mM: After 5 minutes of stabilisation, hearts were perfused with Krebs containing PAG 1mM, then after an additional 5 minutes hearts were perfused with Krebs containing PAG 1mM and L-cysteine 1 mM for the remaining 10 minutes of stabilisation until the first 10 minutes of reperfusion.

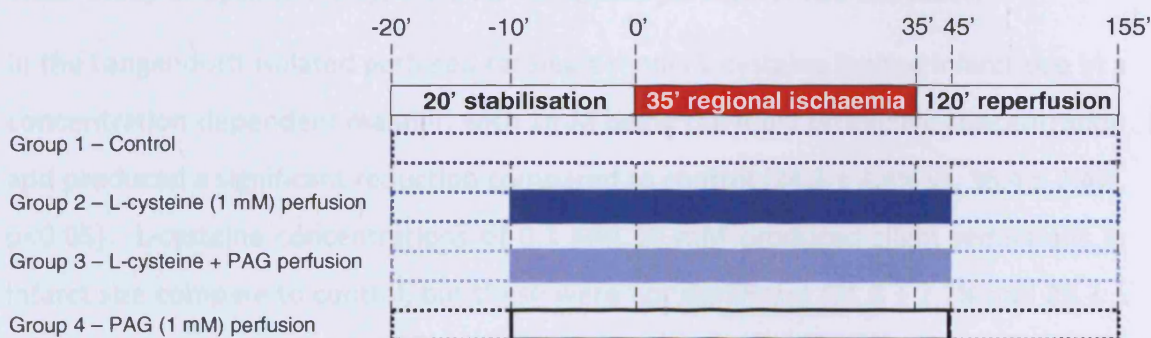


Figure 4.4 Summary of the perfusion protocol for study 3: Effect of PAG on infarct size limitation by L-cysteine. In all treatment groups, 35 minutes of regional ischaemia was induced, after a 20 minute stabilisation period, by tightening the snare on the suture passed around the left main coronary artery. Each heart received 120 min reperfusion following ischaemia. After 10 minutes of reperfusion, the treatment hearts were perfused with Krebs for the duration of the perfusion period.

4.6 Results

4.6.1 Exclusion criteria

To ensure that data was as reproducible within treatment groups and not biased between groups, strict exclusion criteria were used. Hearts were excluded from the study if they failed to obtain a developed pressure less than 50 mmHg during the stabilisation period, had a heart rate less than 200 bpm, and failed to have a flow rate of between 10 and 15 ml/min. Hearts were also excluded on technical grounds during experiments such as pulling the suture out during ischaemia, failing to reduce coronary flow by at least a third during the first 5 minutes of ischaemia, or failing to restore coronary flow at reperfusion.

In total 63 rat hearts were used for the experiments performed in these studies. Of these 63 hearts, 8 were excluded on technical grounds, resulting data from 55 rat hearts being presented in this chapter.

4.6.2 Study 1: Optimum L-cysteine concentration for infarct size limitation

In the Langendorff isolated perfused rat heart model L-cysteine limited infarct size in a concentration-dependent manner, with 1mM being the most protective concentration and produced a significant reduction compared to control ($24.3 \pm 3.4\%$ vs. $36.4 \pm 2.4\%$, $p < 0.05$). L-cysteine concentrations of 0.1 and 10 mM produced slight reductions in infarct size compare to control, but these were not significant ($31.8 \pm 2.2\%$ and $29.3 \pm 3.5\%$ vs. $36.4 \pm 2.4\%$ for 0.1, 10 mM L-cysteine and control respectively) (data summarised in figure 4.5).

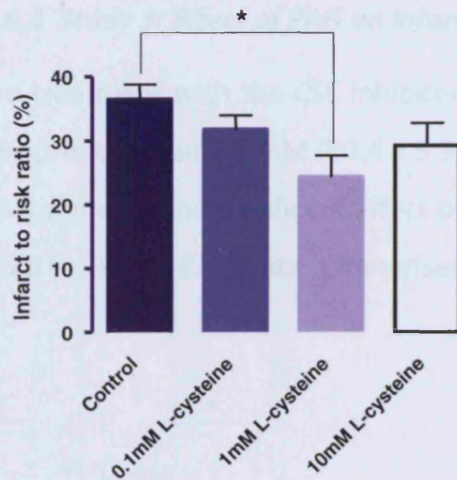


Figure 4.5 Summary of infarct to risk ratios (%) for L-cysteine concentration ranging studies. L-cysteine 1 mM was the most effective at infarct size limitation and was used for subsequent studies. * $p < 0.05$, one-way ANOVA + Newman Keuls post-hoc test.

4.6.3 Study 2: Effect of L-cysteine and P-5'-P on infarct size:

Co-perfusion of L-cysteine 1 mM and P-5'-P 50 μ M, produced no additional protective effect at infarct size limitation compared to L-cysteine alone ($24.3 \pm 3.4\%$ vs. $25.2 \pm 5.1\%$ for L-cysteine alone and L-cysteine + P-5'-P respectively) (data summarised in figure 4.6).

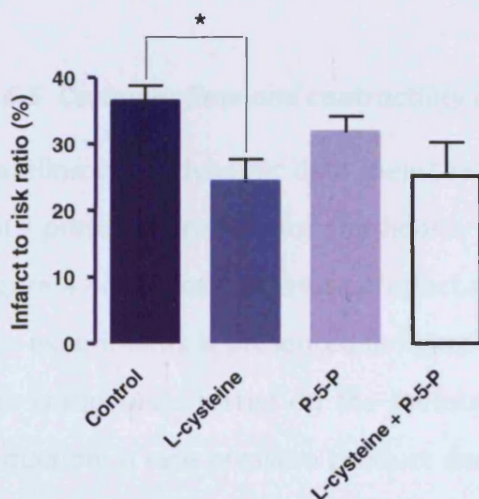


Figure 4.6 Summary of infarct to risk ratios (%) for L-cysteine and P-5'-P perfused hearts. Co-perfusion of L-cysteine and P-5'-P produced no additional cardioprotection over L-cysteine alone. * $p < 0.05$, one-way ANOVA + Newman Keuls post-hoc test.

4.6.4 Study 3: Effect of PAG on infarct size limitation by L-cysteine

Pre-treatment with the CSE inhibitor, PAG 1 mM, partially attenuated the protective effect of L-cysteine 1 mM (30.4 ± 5.9 vs. 24.3 ± 3.4 %) (data summarised in figure 4.6). PAG alone had no significant effect on the size of the infarct compared to control (31.0 ± 5.6 vs. 36.4 ± 2.4) (data summarised in figure 4.7).

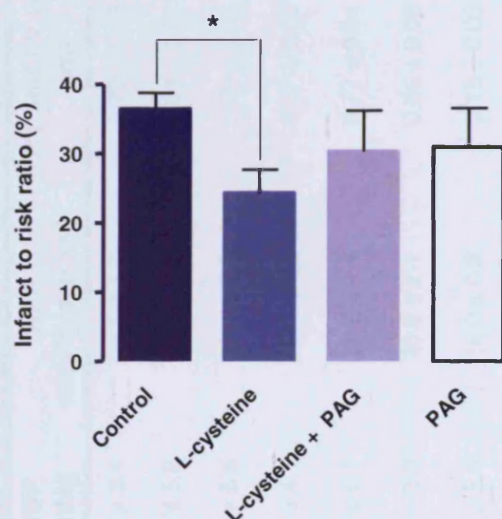


Figure 4.7 Summary of infarct to risk ratios (%) for L-cysteine and PAG perfused hearts. PAG partially attenuated the cardioprotective effects of L-cysteine. * $p < 0.05$, one-way ANOVA + Newman Keuls post-hoc test.

4.6.5 Coronary flow and contractility data

Baseline cardiodynamic data (heart rate, coronary flow rate, developed pressure and rate pressure product for the hearts in each of the three study groups is shown in figure 4.8. The rate pressure product and coronary flow rate of the hearts throughout the experiments is presented in figure 4.8. There were no significant effects of any of the compounds tested on the measured parameters. Regional ischaemia caused a reduction in rate pressure product and coronary flow rate in all treatment groups as expected (as shown in figure 4.9A). Run-down of the hearts is demonstrated by a reduction in rate pressure product in all treatment groups as expected (as shown in figure 4.9B).

Treatment group	n	CFR ml/min	HR BPM	LVDP mmHg	RPP mmHg/min x10 ³	RV and LV vol. cm ³	Risk zone vol. cm ³
Control	12	16.4 ± 1.4	288 ± 8	78.2 ± 5.4	22.5 ± 1.7	0.70 ± 0.03	0.35 ± 0.03
L-cysteine 0.1 mM	4	17.6 ± 1.3	265 ± 22	65.9 ± 5.9	17.6 ± 2.6	0.63 ± 0.07	0.31 ± 0.05
L-cysteine 1 mM	10	17.2 ± 1.1	290 ± 14	79.0 ± 5.6	22.7 ± 1.6	0.78 ± 0.03	0.38 ± 0.04
L-cysteine 10 mM	6	16.3 ± 1.3	251 ± 15	63.8 ± 4.1	15.9 ± 1.2	0.87 ± 0.03	0.49 ± 0.05
PAG 1 mM	6	17.7 ± 1.1	249 ± 8	71.9 ± 5.7	17.7 ± 0.9	0.77 ± 0.04	0.43 ± 0.05
L-cysteine + PAG	6	19.7 ± 0.9	279 ± 24	73.9 ± 3.9	20.6 ± 2.1	0.56 ± 0.03	0.36 ± 0.03
P-5'-P 50 µM	6	14.8 ± 0.7	288 ± 15	55.3 ± 3.0	16.0 ± 0.9	0.76 ± 0.03	0.48 ± 0.03
L-cysteine + P-5'-P	5	15.1 ± 1.4	258 ± 26	83.3 ± 9.0	21.3 ± 2.0	0.77 ± 0.02	0.38 ± 0.04

Figure 4.8 Baseline cardiodynamic data for hearts used in the three studies investigating the role of exogenously stimulated H₂S synthesis on cardioprotection. Abbreviations: LV = left ventricle, RV = right ventricle, CFR = coronary flow rat, HR = heart rate, LVDP = left ventricular pressure, RPP = rate pressure product (HR x LVDP).

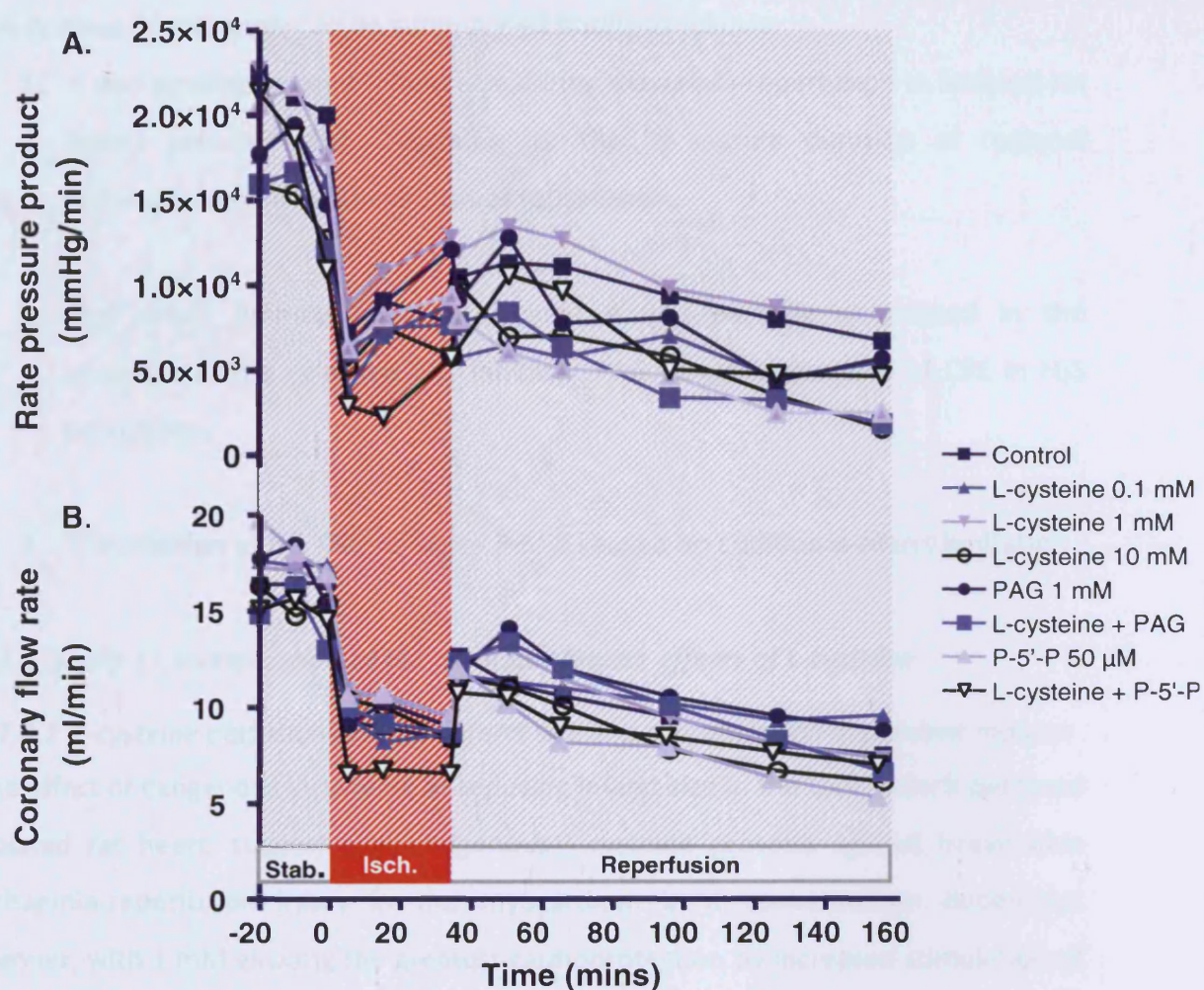


Figure 4.9 Rate pressure product (RPP) (A) and coronary flow rate (CFR) (B) in each of the treatment groups taken throughout the duration of the experiments. Stab.=stabilisation period (20 mins), Isch.=Regional ischaemia (35 mins), Reperfusion (120 mins). Each value shown is the mean from 4-12 individual rats. SEM error bars have been removed for clarity.

4.7 Discussion

The findings of this study can be summarised briefly as follows:

1. It was possible to limit infarct size during ischaemia-reperfusion in isolated rat hearts perfused with L-cysteine for the 35 minute duration of regional ischaemia and initial 15 minutes of reperfusion.
2. The infarct limiting effect of L-cysteine was partially attenuated in the presence of the selective CSE inhibitor PAG, suggesting a role of CSE in H₂S production.
3. The addition of the CSE co-factor P-5'-P caused no additional infarct limitation.

4.7.1 Study 1 - Investigation of the cardioprotective effects of L-cysteine

4.7.1.1 L-cysteine perfusion reduced infarct size in a concentration dependent manner

The effect of exogenous L-cysteine at reducing infarct size in the Langendorff perfused isolated rat heart, suggest that exogenous L-cysteine protects against irreversible ischaemia-reperfusion injury in the myocardium in a concentration dependent manner, with 1 mM eliciting the greatest cardioprotection by increased stimulation of the CSE enzyme to produce H₂S. The observation that 10 mM L-cysteine was less protective than 1 mM L-cysteine, but caused a greater protective effect than 0.1 mM, suggests that higher concentrations of substrate may saturate the enzyme and limit the H₂S production or H₂S is produced at levels that are cytotoxic rather than protective. The cytotoxicity of NaHS at higher concentrations has been implicated by Johansen *et al.* (2006), in which it was reported that exogenously supplied NaHS caused a biphasic concentration dependent reduction in infarct size in the Langendorff perfused rat hearts (0.1-10 μ M). At 10 μ M, the protective effect was diminished and 10 μ M NaHS was associated with cytotoxic, metabolic or other non-specific effects of either the H₂S or its metabolites HS⁻ or S²⁻, or another unknown impurity, that overwhelm the protective action seen at a lower concentration.

4.7.1.2 *L-cysteine may have acted as a free radical scavenger to induce cardioprotection*

An alternative mechanism to explain the cardioprotective effect observed with L-cysteine was that it was acting as a free radical scavenger. It has been reported in the literature that cysteine containing moieties are effective free radical scavengers (Shackebaei *et al.*, 2005). A study by Saez *et al.* (1982) showed that when working with concentrations of L-cysteine 4 mM and above, it was prone to autoxidation with the formation of thiyl and hydroxyl radicals, resulting in membrane damage and depletion of ATP and glutathione. This may explain why L-cysteine 10 mM was produced less cardioprotection than 1 mM. Concentrations of L-cysteine greater than 1 mM have also been shown to cause a decrease in the left ventricular developed pressure under normal conditions (Tani, 1990), which is interesting since there was a tendency, although not significant, for a reduction in the rate pressure product in the L-cysteine 10 mM group during reperfusion.

L-cysteine 0.5 mM has been shown to be cardioprotection in rat hearts subjected to 45 minutes global ischaemia using the same L-cysteine perfusion algorithm as used for the studies presented in this chapter (Shackebaei *et al.*, 2005). Whilst infarct size was not the measured endpoint, a significant increase in functional recovery and preservation of ATP and glutathione levels were reported compared to control. Proposed mechanisms of action include direct scavenging of free radicals or improved antioxidant capacity through the preservation of glutathione levels.

4.7.1.3 *L-cysteine may interact with NO to form S-nitrothiols*

L-cysteine, at concentrations in the millimolar range, has also been shown to interact with nitric oxide to form S-nitrothiols (Stamler, 1994). The formation of these S-nitrothiol complexes act as nitric oxide carriers and stabilise the activity of nitric oxide free radicals. Therefore an additional mechanism by which L-cysteine may have induced cardioprotection in these experiments was by the formation of S-nitrothiols stabilising the large amount of free nitric oxide free radicals which would be formed at reperfusion. It could also be speculated that L-cysteine may also interact with other free radicals formed at reperfusion such as the superoxide anion (O_2^-) and the hydroxyl

radical ($\cdot\text{OH}$), formed from the reintroduction of oxygen, acting to stabilise them and resulting in cardioprotection.

However, a recent study by Khanna *et al.* (2008) showed that preconditioning with N-acetyl cysteine, an analogue of L-cysteine, 1 mM attenuated physiological and pharmacological preconditioning in isolated rat hearts, and also inhibited remote preconditioning in an *in vivo* rat model. Interestingly, a cysteine containing nitric oxide donor, SP/W-518, has been shown to attenuate post-ischaemia myocardial injury in isolated perfused rabbit hearts (Liu *et al.*, 1998). However, an analogue compound of SP/W-518, SPW/-6373, lacking the nitric oxide moiety, induced no cardioprotection. These findings suggest that cysteine may not have a cardioprotective action as many have proposed, and can in fact be inhibitory to the cardioprotective actions of other stimuli. However, the findings from the study presented in this chapter oppose these findings, but differences in experimental protocols and the fact that N-acetylcysteine as opposed to L-cysteine was used may also explain these contradictory findings.

4.7.2 Study 2 - Effect of the addition of P-5'-P to the cardioprotective effect of L-cysteine

4.7.2.1 Perfusion with P-5'-P produced no additional cardioprotection

The addition of pyridoxal 5'phosphate, the cofactor of the CSE enzyme, produced no additional reduction in infarct size when used in combination with L-cysteine, compared to L-cysteine 1mM alone. In fact it slightly increased the infarct size and as a result there was not a significant cardioprotective effect observed. This suggests that sufficient cofactor was already present in the myocardium to enable maximal production of H₂S. Alternatively this finding suggests that not a high enough concentration of P-5'-P was tested, resulting in limited H₂S production. The latter explanation seems unlikely though since the concentration tested was based on an estimate of physiological levels. Perhaps a better explanation is that the P-5'-P was unable to cross internal membranes and reach the site of the H₂S synthesising enzymes and therefore unable to further increase H₂S production. This may have been due to the size or the charge on the compound impeding its access.

Some interesting clinical studies have been conducted with pyridoxal-5'-phosphate to investigate its cardioprotective actions. In a phase II pilot study it has been shown that patients pre-treated with P-5'-P monohydrate (MC-1) prior to percutaneous coronary intervention, had a significant reduction in myocardial ischaemic injury, determined by measurement of the area under the curve of creatine kinase, compared to placebo control (Kandzari *et al.*, 2003). These findings suggest that P-5'-P induces cardioprotection and may therefore be a useful therapeutic intervention prior to percutaneous coronary intervention. Further trials in patients at risk from developing myocardial ischaemia, infarction or reperfusion injury are required to further explore this potential therapeutic intervention.

P-5'-P levels measured in French-Canadian patients suffering from coronary artery disease, have been shown to be significantly reduced compared to healthy subjects (Dallery *et al.*, 1995). It has been suggested that reduced P-5'-P levels may be a contributing factor to elevated homocysteine levels observed in patients with coronary artery disease. Homocysteine levels have been identified as a risk factor for atherosclerotic disease although the link is poorly understood (Verhoef *et al.*, 1997). A proposed atherogenic mechanism involves the inhibition of growth of endothelial cells and stimulation of smooth cell proliferation by homocysteine, resulting in thickening of arterial walls. Therefore patients suffering from coronary artery disease could potentially be treated with P-5'-P supplements, to lower homocysteine levels and reduce atheroma formation.

In view of these findings, it suggests that P-5'-P alone has the ability to produce cardioprotection. However, in the studies presented in this chapter it showed no cardioprotective effect when tested alone or in combination with L-cysteine. A possible explanation for the lack of cardioprotection on its own is that whilst it may have the potential to play an important role in cardioprotection, it may have more of an involvement in preventing the progression of atherosclerosis and reducing associated damage to the heart in a more indirect manner. Therefore in a model of acute myocardial infarction direct cardioprotection is unlikely to occur.

4.7.3 Study 3 - Effect of cardioprotective effect of L-cysteine in the presence of PAG

4.7.3.1 PAG attenuated the cardioprotective effect of L-cysteine

The finding that the protective effect of L-cysteine was partially attenuated in the presence of PAG suggests that the cardioprotective effect of L-cysteine may at least be partially mediated through the activation of the enzyme CSE, resulting in H₂S generation and subsequent cardioprotection. The other small amount of remaining cardioprotection observed with L-cysteine in the presence of PAG may be due to L-cysteine's ability to act as a free radical scavenger or the generation of H₂S from another source.

The use of a CSE inhibitor with a different mechanism of action would help to confirm this, since it may be more potent and therefore able to totally abolish the cardioprotection. The use of a specific CBS enzyme inhibitor, such as aminooxyacetic acid, would also be interesting, since it has been reported that in the myocardium CSE is the main enzyme responsible for H₂S production (Tang *et al.*, 2006a) and this could be used to confirm these findings. This does not rule out a contribution of H₂S from the CBS enzyme, although findings by Chen *et al.* (1999) reported that there was no activity or expression of CBS in human atrium and ventricles. This suggests that MST, the third H₂S producing enzyme, may be responsible for the small protective effect observed during ischaemia when the CSE enzyme would be expected to be inhibited by PAG. The use of a selective CBS inhibitor (e.g. aminooxyacetic acid) in combination with a selective CSE inhibitor (PAG) would enable the potential contribution of the MST enzyme to H₂S production to be determined. This may provide evidence as to whether MST is another H₂S synthesising enzyme that could be targeted for protection against ischaemia- reperfusion injury.

4.7.4 Further studies

The Langendorff isolated perfused rat heart model could be developed further by using a constant flow system to investigate the effect of L-cysteine perfusion and whether it effects coronary tone. A reduction in coronary tone, may indicate the generation of H₂S, providing a greater insight into the cardioprotective mechanism of action of L-cysteine perfusion. From the studies conducted with L-cysteine it was not possible to determine when L-cysteine was inducing its cardioprotective since hearts were perfused over ischaemia and early reperfusion. Perfusing hearts with L-cysteine at different time points and observing when it produces its greatest cardioprotection, would determine if it has an optimum time point of action. Experiments performed in Chapter 5 investigated the effect of perfusing the heart with an H₂S donor compound and observing the effect on infarct size, as well as measuring the H₂S tissue concentration in rat hearts stimulated with L-cysteine, and the effect of ischaemia-reperfusion on the maximal H₂S synthesising capacity of the myocardium.

4.8 Conclusion

Taken together, the findings of these three studies show that exogenous L-cysteine exhibited a concentration dependent reduction in infarct size, with a concentration of 1 mM eliciting the greatest protection. This cardioprotective effect of L-cysteine was not enhanced in the presence of CSE's co-factor pyridoxal-5-phosphate. The protective effect of L-cysteine was attenuated in the presence of the selective CSE enzyme inhibitor PAG, suggesting that exogenous L-cysteine may have stimulated increased H₂S production via the CSE enzyme. To determine if increased H₂S production did occur after stimulation with L-cysteine, a concentration determining assay could be performed. This would assist in deciphering the mechanism of cardioprotection induced by L-cysteine, as currently there remains a level of uncertainty as to whether L-cysteine was acting as a free radical scavenger to induce cardioprotection or whether its cardioprotective action was through the generation of H₂S gas, and if it was through the generation of H₂S, which enzyme(s) was responsible.

Chapter 5: Measurement of the maximal H₂S synthesising capacity and tissue concentrations of H₂S, in hearts subjected to ischaemia-reperfusion or L-cysteine perfusion

5.1 Introduction

As described in detail in the Chapter 1 the myocardium predominantly expresses the H₂S synthesising enzyme CSE. This was investigated further in Chapter 3 by characterising the mRNA and protein expression, and also in Chapter 4 where stimulated H₂S production caused infarct size limitation. Measurement of the stimulated activity of this enzyme can be determined experimentally, as well as endogenous tissue concentrations of H₂S and would provide a greater understanding of the role of H₂S and the effect of ischaemia-reperfusion its production. Stipanuk and Beck (1982) outline a method for the measurement of exogenously stimulated enzymatic production of H₂S gas from homogenised tissue samples which has been widely adopted. The technique relies on using the substrate L-cysteine and co-factor, P-5'-P, to stimulate the H₂S generating enzymes to produce H₂S gas. The resulting gas is then trapped on zinc acetate soaked filter paper. The trapped gas then causes a colorimetric change in a ferric chloride solution as the iron is oxidised. The resulting solution is measured spectrophotometrically and the use of H₂S standards prepared from the donor NaHS enables a standard curve and the concentration of the sample to be determined. Examples of the broad spectrum of tissues used to investigate stimulated enzymatic production of H₂S are shown in figure 5.1. This also highlights the diversity of the units of expression for the maximal enzyme synthesising capacity, and also the limited number of studies investigating the myocardium.

Species	Tissue	Conditions	H ₂ S production	Reference
Rat	Liver	280 mM L-cys, 3 mM P-5'-P	0.403μmol/min/g wet tissue	(Stipanuk <i>et al.</i> , 1982)
	Kidney		0.192μmol/min/g wet tissue	
	Liver	160 mM L-cys, 2 mM P-5'-P, 3 mM 2-oxo, 3 mM dithio	2.31μmol/min/g wet tissue	
	Kidney		1.96μmol/min/g wet tissue	
	Liver	2 mM L-cys, 0.05 mM P-5'-P	0.017μmol/min/g wet tissue	
	Kidney		0.013μmol/min/g wet tissue	
	Liver	2 mM L-cys, 0.05 mM P-5'-P, 0.5 mM 2-oxo, 0.05 mM dithio	0.019μmol/min/g wet tissue	
	Kidney		0.018μmol/min/g wet tissue	
Rat	Myocardium	10 mM L-cys, 2 mM P-5'-P	18.64nmol/min/g protein	(Geng <i>et al.</i> , 2004c)
Rat	Liver	10 mM L-cys, 1mM P-5'-P	40 nmol/mg protein	(Mok <i>et al.</i> , 2004)
	Kidney		17 nmol/mg protein	
Rat	Thoracic aorta	10 mM L-cys, 2 mM P-5'-P	4 pmol/min/mg wet tissue	(Hui <i>et al.</i> , 2003)
	Pulmonary artery		5 pmol/min/mg wet tissue	
	Mesenteric artery		6 pmol/min/mg wet tissue	
	Tail artery		8 pmol/min/mg wet tissue	
Mouse	Liver	10 mM L-cys, 2 mM P-5'-P	0.59 nmol/mg protein	(Li <i>et al.</i> , 2005)
	Kidney		0.127 nmol/mg protein	
Rat	Mesenteric artery bed	1 mM L-cys	22.5 nM/min/g wet tissue	(Cheng <i>et al.</i> , 2004)
Rat	Ileum	10 mM L-cys, 2 mM P-5'-P	20.3 nMol/min/g protein	(Hosoki <i>et al.</i> , 1997)
	Portal vein		19.6 nMol/min/g protein	
	Thoracic aorta		33.7 nMol/min/g protein	
Marine invertebrate Tissue (clam and lugworm)	Gill	10 mM L-cysteine, 2 mM P-5'-P	0.25 nmol/g/min	(Julian <i>et al.</i> , 2002)
	Foot		0.045 nmol/g/min	
	Mantle		0.047 nmol/g/min	
	Body wall		0.37 nmol/g/min	

Figure 5.1 Examples of the broad spectrum of tissues used to investigate stimulated enzymatic production of H₂S. Note the diversity of the units of expression for the maximal enzyme synthesising capacity, and also the limited number of studies investigating the myocardium.

Stipanuk and Beck's method has subsequently been adapted by Mok *et al.* (2004) to enable the amount of H₂S contained within a tissue sample to be measured. It relies on the same principle of trapping the gas and the H₂S undergoing a colour change to determine the concentration. Whilst relatively simple in principle, these assays are labour intensive having multiple steps with opportunity for error to occur. However, despite this limitation they appear to be reproducible and have widespread use in the literature.

5.2 Aim

The aim of the studies conducted in this chapter was to investigate the endogenous H₂S synthesising ability of the rat myocardium.

5.3 Hypotheses

The hypotheses tested in this series of experiments were as follows:

1. Exogenous stimulation of H₂S synthesising enzymes within the heart will enable the maximal production rate of H₂S to be measured
2. The maximal activity of H₂S synthesising enzymes will be altered in hearts subjected to ischaemia and reperfusion
3. The H₂S concentration will be altered in left ventricle samples from hearts subjected to ischaemia and reperfusion

5.4 Objectives

The validity of these hypotheses was tested through the following specific objectives:

1. To develop and apply an assay to enable the maximal H₂S synthesising ability of the heart tissue to be measured, after exogenous stimulation.
2. To develop and apply an assay to enable the H₂S concentration in the myocardial tissue to be released and measured.

5.5 Methods

Two series of experiments were undertaken (as summarised in figure 5.2). In the first the effects of ischaemia-reperfusion were investigated in Langendorff perfused rat hearts. In the second the effects of L-cysteine stimulated H_2S production were investigated. Samples obtained were then used in assays to measure maximal H_2S enzyme synthesising capacity or to determine the H_2S concentration in the myocardial tissue.

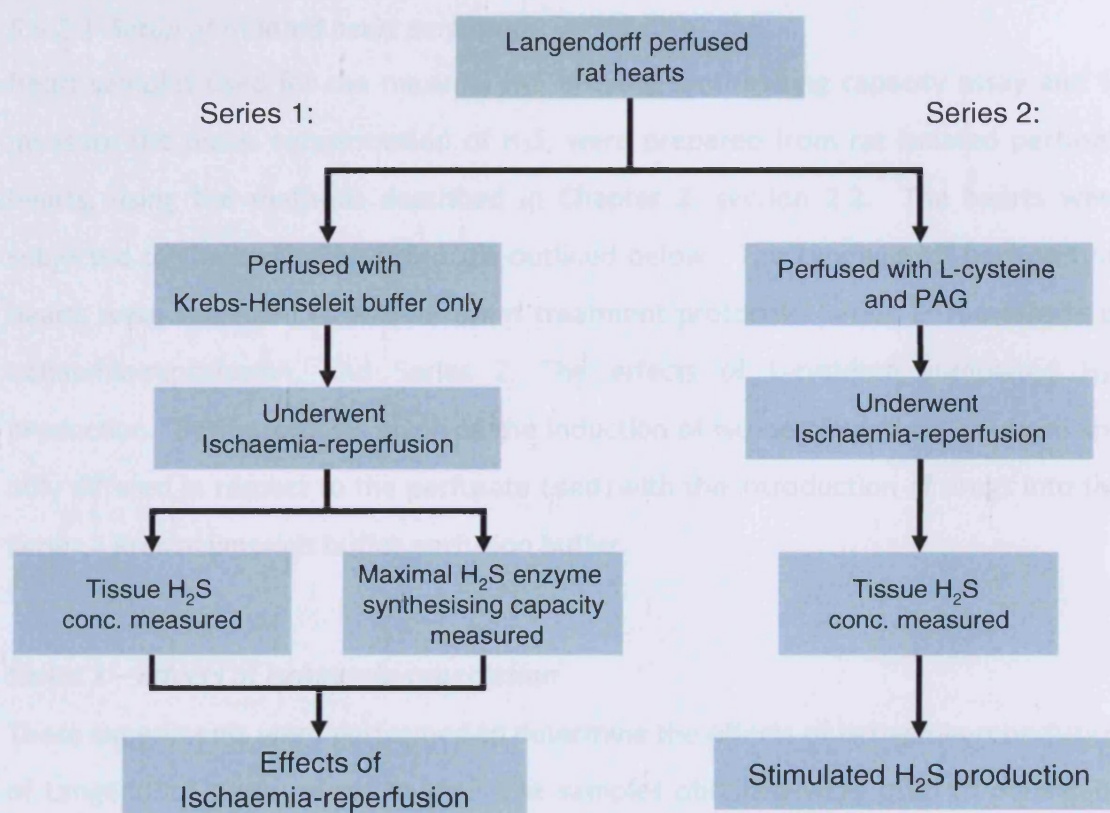


Figure 5.2 Flow diagram to show the two series of experiments that were performed to prepare Langendorff perfused hearts samples to investigate either the specific effects of ischaemia-reperfusion (Series 1) or the effect of exogenously stimulated H_2S production (Series 2). Samples from Series 1 were used to measure the H_2S concentration or the maximal H_2S synthesising capacity in the myocardial tissue. Samples from Series 2 were used only to measure the H_2S concentration in the myocardial tissue.

5.5.1 Source of compounds

The H₂S donor NaHS was purchased from Acros Organics, UK. The CSE substrate L-cysteine and the CSE inhibitor DL-propargylglycine (PAG) were purchased from Sigma-Aldrich, UK. All compounds were dissolved in Krebs-Henseleit buffer and therefore required no vehicle controls.

5.5.2 Preparation of Langendorff perfused rat heart

5.5.2.1 Setup of isolated heart perfusion:

Heart samples used for the maximal H₂S enzyme synthesising capacity assay and to measure the tissue concentration of H₂S, were prepared from rat isolated perfused hearts, using the methods described in Chapter 2, section 2.2. The hearts were subjected to the treatment protocols outlined below. The Langendorff perfused rat hearts were subjected to two different treatment protocols: Series 1: The effects of ischaemia-reperfusion, and Series 2: The effects of L-cysteine stimulated H₂S production. Both protocols involved the induction of ischaemia and reperfusion, and only differed in respect to the perfusate used, with the introduction of drugs into the Series 2 Krebs-Henseleit buffer perfusion buffer.

Series 1 – Effects of ischaemia-reperfusion

These experiments were performed to determine the effects of ischaemia-reperfusion of Langendorff perfused rat hearts. The samples obtained were used to determine the maximal H₂S synthesising enzyme capacity and also for the measurement of the basal concentration of H₂S in the myocardial tissue.

Treatment protocols for this study are illustrated in Figure 5.3.

Group 1 – Baseline control: Hearts were perfused with Krebs-Henseleit buffer for the duration of the 20 minute stabilisation period.

Group 2 – Regional ischaemia: As per group 1, except that at the end of the stabilisation period hearts were subjected to 35 minutes regional ischaemia.

Group 3 - Regional ischaemia and reperfusion: As per group 2 with the addition of 15 minutes reperfusion following 35 minutes regional ischaemia.

At the end of the perfusion experiments, hearts from all experimental groups were briefly washed in PBS, blot dried, the left and right ventricles separated and snap frozen in liquid nitrogen. The samples were stored at -80°C prior to measurement of the H_2S concentration or maximal H_2S synthesising enzyme capacity in the myocardium.

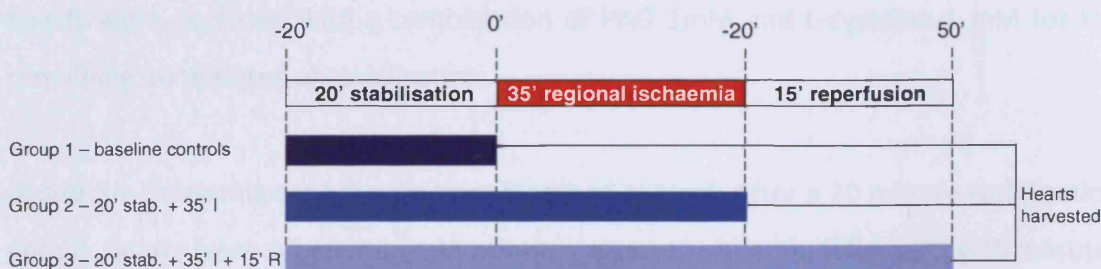


Figure 5.3 Summary of the Series 1: Krebs-Henseleit buffer perfused hearts protocols. Stabilisation period consisted of 20 minutes of perfusion with Krebs-Henseleit buffer. Hearts were then either harvested (group1), went on to receive 35 minutes regional ischaemia (group 2), or received 35 minutes ischaemia followed by 15 minutes reperfusion before harvested. (group 3). At time of harvesting, hearts were removed from the cannula and dissected into left and right ventricle. The sections were then washed briefly in PBS, blot dried and snap frozen in liquid nitrogen, before storage at -80°C . Samples from this protocol were used for the determination of the maximal H_2S synthesising enzyme capacity and also for the measurement of the basal H_2S concentration in the myocardial tissue.

Series 2 – L-cysteine perfused hearts

A second series of experiments were performed to determine the effect of L-cysteine stimulated H_2S production on the concentration of H_2S in the myocardial tissue.

Treatment protocols for this study are illustrated in Figure 5.4.

Group 1 – Baseline control: Hearts were perfused with Krebs-Henseleit buffer for the 20 minute stabilisation period.

Group 2 – L-cysteine 1 mM: As per group 1, except that for the final 10 minutes of stabilisation hearts were perfused with Krebs-Henseleit buffer containing L-cysteine 1mM.

Group 3 - PAG 1 mM: As per group 2, except that hearts were perfused with Krebs-Henseleit buffer containing PAG 1 mM for the final 10 minutes of stabilisation.

Group 4 - L-cysteine 1 mM and PAG 1 mM: After 5 minutes of stabilisation hearts were perfused with Krebs-Henseleit buffer containing PAG 1 mM after a further 5 minutes, hearts were perfused with a combination of PAG 1mM and L-cysteine 1 mM for the remaining 10 minutes of stabilisation.

Group 5 – Ischaemia-reperfusion time matched control: After a 20 minute stabilisation period, hearts were subjected to 35 minutes regional ishaemia followed by 15 minutes reperfusion. For the duration of the experiment hearts were perfused with Krebs-Henseleit buffer.

Group 6 - L-cysteine 1 mM: As per group 1, except that after 10 minutes of stabilisation and until 10 minutes reperfusion hearts were perfused with Krebs-Henseleit buffer containing L-cysteine 1 mM.

Group 7 - PAG 1 mM: As per group 6, except hearts were perfused with PAG 1 mM.

Group 8 - L-cysteine 1 mM and PAG 1 mM: After 5 minutes of stabilisation hearts were perfused with PAG 1 mM, after a further 5 minutes hearts were perfused with L-cysteine and PAG 1 mM until 10 minutes reperfusion.

At the end of the perfusion experiments, hearts from all experimental groups were briefly washed in PBS, blot dried, the left and right ventricles separated and snap frozen in liquid nitrogen. The samples were stored at -80 °C prior to measurement of the concentration of H₂S.

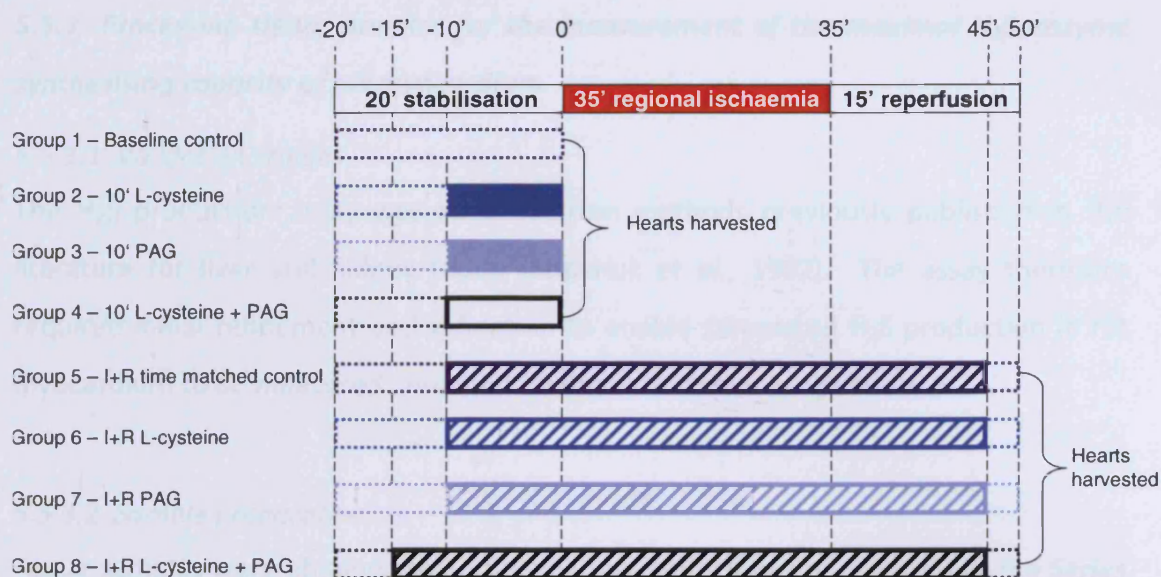


Figure 5.4 Summary of the Series 2 - Drug perfused hearts protocols. Once cannulated, hearts were left to stabilise for at least 10 minutes until a steady sinus rhythm was obtained. The experimental stabilisation period lasted 20 minutes during which time all hearts were perfused with Krebs-Henseleit buffer for the first 10 minutes. For the remaining 10 minutes of stabilisation, hearts were perfused with L-cysteine, PAG or a combination of drugs. Time-matched control hearts were perfused with Krebs for the remainder of the experiment. Hearts were then either harvested (groups 1-4) or went on to receive ischaemia and reperfusion (groups 5-8). When combinations of L-cysteine and PAG were used, the PAG was perfused after 5 minutes of the stabilisation period to give it a chance to act. Regional ischaemia was induced for 35 minutes and followed by 15 minutes of reperfusion. For the final 5 minutes of reperfusion hearts were perfused with Krebs-Henseleit buffer alone. Hearts were then harvested at this point in the protocol (groups 5-8). At the end of perfusion the sections were washed briefly in PBS, blot dried and snap frozen in liquid nitrogen, before storage at -80°C . Samples from this protocol were used for the measurement of H_2S concentrations in the myocardial tissue.

At the end of the perfusion experiments, hearts from all experimental groups were briefly washed in PBS, blot dried, the left and right ventricles separated and snap frozen in liquid nitrogen. The samples were stored at -80°C prior to the measurement of the H_2S concentration in the tissue.

5.5.3 Processing tissue samples for the measurement of the maximal H₂S enzyme synthesising capacity of rat myocardium

5.5.3.1 Validation studies

The H₂S production assay was adapted from methods previously published in the literature for liver and kidney tissue (Stipanuk *et al.*, 1982). The assay therefore required initial refinement and validation to enable stimulated H₂S production in rat myocardium to be measured. Preliminary data are shown in Appendix 2.

5.5.3.2 Sample preparation

Tissue samples were obtained by subjecting Langendorff perfused hearts to the **Series 1 – Effects of ischaemia-reperfusion** experimental protocol as outlined in figure 5.1 of this chapter. 10% (w/v) of left ventricle and right ventricle homogenates were prepared by suspending crushed tissue samples, in ice cold sodium phosphate buffer (pH 7.4) (see Appendix 3 for composition). Liver samples obtained from rats were also tested as a positive control. The homogenates prepared were stored on ice until assayed.

5.5.3.3 Protein determination in tissue samples

The protein concentrations of the tissue samples were determined using the BCA assay as described in Chapter 2, section 2.3. Protein determination has enabled the production rates of H₂S per g of protein to be calculated and allows a more accurate comparison of the maximal H₂S synthesising capacity between tissues.

5.5.3.4 H₂S production and collection

25 ml wide neck Erlenmeyer flasks were used to perform the H₂S production assay and were set up such that a 2 ml Eppendorf tube was suspended in each flask using a thread. The thread was secured to the neck of the flasks using a thin strip of parafilm, enabling the Eppendorf tube to be securely suspended inside the flask. A 15:1 (v/v) mixture of 1 % (w/v) zinc acetate solution and 12 % (w/v) sodium hydroxide solution was added to the suspended Eppendorf tube. A piece of Whatman 0.34 mm chromatographic paper (2 x 2.5 cm), folded into a fan shape, was placed in the

Eppendorf tube. The filter paper absorbed the zinc acetate and sodium hydroxide solution enabling the trapping of H_2S gas. 500 μl of sodium phosphate buffer (pH 7.4), 100 μl of a 12.1 mg/ml stock of L-cysteine, and 100 μl of a 5.3 mg/ml stock of pyridoxal 5'phosphate was added to the 25 ml Erlenmeyer flask. The final assay concentrations of L-cysteine and pyridoxal 5'phosphate were 10 mM and 2 mM respectively. The flasks were then flushed with N_2 for 20 seconds and 300 μl of tissue homogenate was added. In the case of control samples 300 μl of sodium phosphate buffer was added instead of tissue homogenate. The total volume of the reaction mixture in the assay was 1 ml. The flasks were double sealed with parafilm and incubated in a covered water bath at 37 °C with regular mixing of the contents. The reaction was stopped after 15 minutes by the injection of 0.5 ml of 50 % (w/v) trichloroacetic acid. The flasks were then sealed with another layer of parafilm, before being returned to the water bath for 60 minutes to allow complete trapping of the gas. During this period the content of the flasks were regularly mixed. The content of the Eppendorf tube was thoroughly washed into a test tube containing 3.5 ml of dH_2O . 400 μl of N,N-dimethyl-p-phenylenediamine sulphate (20 mM) in 7.2 M HCl was added. Then 400 μl of FeCl_3 (30 mM) in 1.2 M HCl was added and the test tube gently shaken. After 10 minutes a blue colour formed to indicate the presence of H_2S gas. The absorbance of the solution was read on a spectrophotometer at 670 nm (Jenway Model 6305). The spectrophotometer was calibrated on the day of experiment to the 0 μM H_2S sample (dH_2O).

5.5.3.5 Standard curve to NaHS

Standard curves to NaHS, an H_2S donor, were produced to enable equivalent H_2S concentrations to be determined by the tissue samples in the assay. By calibrating to the 0 μM H_2S sample the standard curve for NaHS passed through the origin. The use of NaHS allowed the concentration of H_2S to be determined more precisely than if H_2S gas was simply bubbled through water. When NaHS dissociates in water it forms Na^+ and HS^- . The HS^- then associates with H^+ to form H_2S . In solution at pH 7.4 and 37 °C, approximately one third of H_2S exists in the undissociated form H_2S and the remaining two thirds exists as HS^- which is at equilibrium with H_2S . Therefore when making a

stock of H_2S from a donor it is important to take into account that only one third of the H_2S will be in the undissociated form (Cheng et al 2004). However, it was assumed that if this is how the H_2S behaved when made from the donor, the H_2S released from the tissue would behave in the same manner. Therefore the NaHS concentration was not adjusted to compensate for this. A 600 μM final assay concentration stock of NaHS was prepared fresh on the day of experimentation. This was necessary because it had been identified in preliminary studies that frozen aliquots of NaHS were not stable at -20°C . The 600 μM final assay concentration (FAC) stock was then diluted to produce 400 μM , 100 and 0 μM FAC stocks. 300 μl samples of each of these concentrations were added in place of tissue homogenates. This meant that four reaction flasks containing the NaHS standards could be run alongside the sample flasks. A standard curve for NaHS in each experiment ensured an accurate estimation of H_2S production and also acted as an internal control.

Standard curves to NaHS were plotted in Excel and the equation of the line used to predict the concentration of H_2S produced in the tissue samples based on the absorbance values obtained.

5.5.4 Measurement of the H₂S concentration in the left ventricle

The following method for the measurement of the H₂S concentrations within myocardial tissue was adapted from Mok *et al.* (2004). Tissue samples were obtained by subjecting Langendorff perfused hearts to the Series 1 – Effects of ischaemia-reperfusion and Series 2 – L-cysteine perfused hearts experimental protocols as outlined in figures 5.2 and 5.2 in this chapter. 40% (w/v) tissue homogenates of left ventricle samples were prepared, by initially homogenising 40 mg snap frozen tissue samples with a mortar and pestle. The mortar was cooled on dry ice, and filled with liquid nitrogen to prevent degradation of the sample. 1 ml of phosphate buffer was added and the tissue samples further homogenised, with a mortar and pestle, until a smooth homogenate was obtained (approx. 5 minutes of grinding). The resulting homogenate was spun at 13000 rpm for 5 minutes and 500 µl removed and added to 750 µl TCA (10% w/v). The resulting precipitate was spun at 13000 rpm for 5 minutes. 500 µl of the supernatant was removed and placed in an Eppendorf tube. 150 µl of Zinc acetate (1% w/v), 100 µl N,N-dimethyl-p-phenylenediamine sulphate (20 mM) in 7.2 M HCl, and 133 µl FeCl₃ (30 mM) in 1.2 M HCl was then added. After 10 minutes, a 20 µl aliquot of each tissue sample was placed in duplicate in a 96 well plate. The absorbance was read at 620 nM. The absorbance values were then compared to an NaHS standard curve conducted in the same plate (for details of the NaHS standard curve see section 5.5.3.5). A BCA assay was also performed to enable the protein concentrations of the samples to be determined (see Chapter 2, section 2.3).

5.6 Results

5.6.1 Exclusion criteria

In total 73 rat hearts were used for the experiments performed in these studies. Of these 73 hearts, 11 were excluded on technical grounds. Data from 62 rat hearts are presented in this chapter taking into account that some tissue samples were used for both series of experiments.

5.6.2 Effects of ischaemia-reperfusion on the maximal H_2S enzyme synthesising capacity

Exogenously stimulated H_2S production rates were measured as an indication of the maximal H_2S synthesising capacity of left and right ventricle samples from Langendorff perfused hearts. In baseline stabilised rat hearts (group 1) the maximal H_2S synthesising capacity did not differ between the left and right ventricle (1.21 ± 0.14 vs. $0.866 \pm 0.37 \mu\text{mol/g/min}$). There was an increase, although not significant, in the H_2S production in both the left and right ventricles above baseline during ischaemia (group 2). The ischaemic left ventricle produced 1.70 ± 0.30 vs. $1.21 \pm 0.14 \mu\text{mol/g/min}$ for control, whilst the right ventricle produced 1.83 ± 0.5 vs. $0.866 \pm 0.37 \mu\text{mol/g/min}$ for control. However, in hearts subjected to ischaemia and reperfusion (group 3), the left ventricle showed a significant reduction ($p < 0.05$) in the maximal H_2S synthesising capacity, whilst the right ventricle remained unaffected (0.396 ± 0.066 vs. $1.40 \pm 0.28 \mu\text{mol/g/min}$) ($p < 0.05$) (data summarised in figure 5.5).

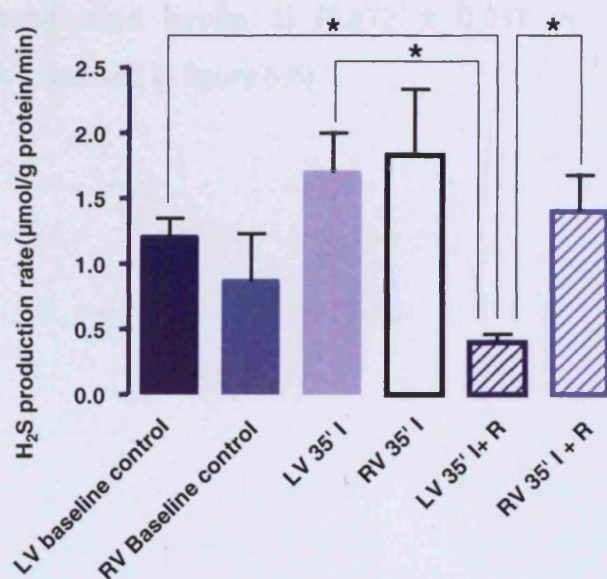


Figure 5.5 Maximal H_2S synthesising capacity in left and right ventricle samples from rat isolated Langendorff perfused hearts. The hearts received either 20 minutes of perfusion, 20 minutes of perfusion followed by 35 minutes ischaemia, or 20 minutes of perfusion followed by 35 minutes ischaemia and 15 minutes reperfusion. The maximal H_2S synthesising ability of the samples was measured in a biochemical assay by stimulating the H_2S synthesising enzyme, in homogenates of the tissue samples, with the substrate L-cysteine and the co-factor pyridoxal 5'phosphate. Each value shown is the mean \pm SEM from 6 individual hearts. * $p < 0.05$. (1-way ANOVA).

5.6.3 Measurement of the H₂S concentration in left ventricle

Left ventricle tissue samples from Langendorff perfused rat hearts that underwent Series 1 and 2 perfusion protocols (outlined in figures 5.3 and 5.4), were assayed to determine the H₂S concentration.

5.6.3.1 Series 1 – Effects of ischaemia-reperfusion

Measurement of the basal H₂S concentration

There was a significant increase ($p < 0.05$) in the basal H₂S concentration in Krebs-Henseleit buffer perfused hearts that underwent 20 minutes of stabilisation followed by 35 minutes of regional ischaemia (group 2) compared to hearts that had only undergone 20 minutes of stabilisation (group 1) (0.653 ± 0.052 vs. 0.410 ± 0.12 $\mu\text{mol/g}$ protein). There was a very significant ($p < 0.01$) increase in the basal H₂S concentration in hearts that underwent 20 minutes of stabilisation, 35 minutes of regional ischaemia and 15 minutes of reperfusion (group 3) compared to hearts subjected to 20 minutes stabilisation (group 1) (0.872 ± 0.037 vs. 0.410 ± 0.12 $\mu\text{mol/g}$ protein) (data summarised in figure 5.6).

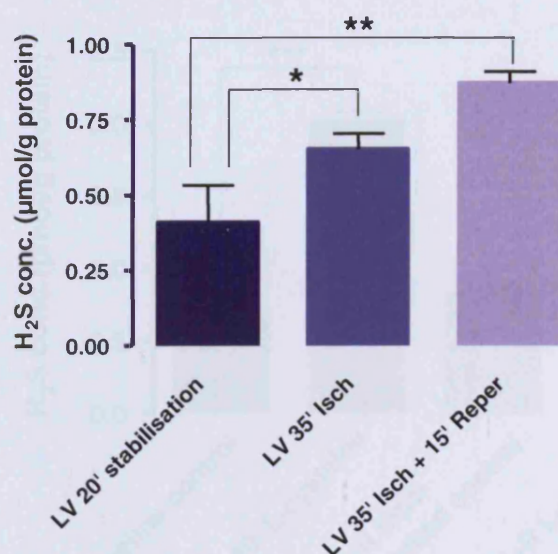


Figure 5.6 Comparison of the basal H₂S concentration in left ventricle samples from rat isolated heart samples perfused with Krebs-Henseleit buffer, either subjected to 20 minutes of stabilisation, 20 minutes of stabilisation plus 35 minutes of regional ischaemia, or 20 minutes of stabilisation, 35 minutes of regional ischaemia and 15 minutes of reperfusion. These results acted as time matched controls for subsequent drug perfused heart experiments. The basal H₂S concentrations of the samples were measured in a biochemical assay, involving release and trapping of the gas. Each value shown is the mean \pm SEM from 6 individual hearts. * $p < 0.05$, ** $p < 0.01$ (1-way ANOVA).

5.6.3.2 Series 2 – Effects of L-cysteine perfused hearts

Measurement of the H₂S concentration in L-cysteine perfused hearts

Rat hearts perfused with CSE/CBS substrate L-cysteine (1 mM) during the 20 minute stabilisation period, showed a highly significant ($p < 0.001$) increase in the H₂S concentration, compared with baseline control (2.01 ± 0.069 vs. 0.639 ± 0.16 $\mu\text{mol/g}$ protein). Hearts perfused with L-cysteine (1 mM) during ischaemia and reperfusion, showed no significant change in the H₂S concentration from time-matched control (0.759 ± 0.11 vs. 0.825 ± 0.043 $\mu\text{mol/g}$ protein) (data summarised in figure 5.7).

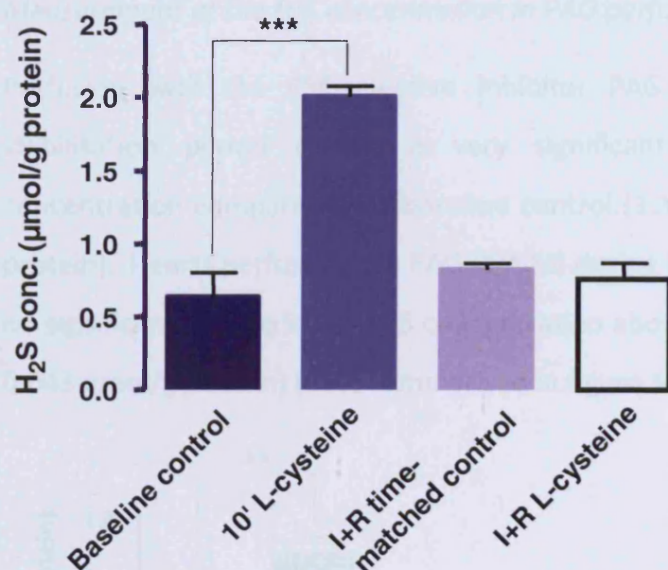


Figure 5.7 Comparison of the H₂S concentration in left ventricle samples from rat isolated heart samples perfused with L-cysteine (1 mM) for either the final 10 minutes of stabilisation, or the final 10 minutes of the stabilisation period, and 35 minutes of regional ischaemia and 10 minutes reperfusion. The Baseline control and time matched control were perfused with Krebs-Henseleit buffer for the duration of the experiment. The H₂S concentrations of the samples were measured in a biochemical assay, involving release and trapping of the gas. Each value shown is the mean \pm SEM from 5-6 individual hearts. ***P<0.01. (1-way ANOVA).

Measurement of the H₂S concentration in PAG perfused hearts

Perfusion with the CSE-selective inhibitor PAG (1 mM) during the 20 minute stabilisation period caused a very significant ($p < 0.01$) increase in the H₂S concentration compared with baseline control (1.32 ± 0.086 vs. 0.639 ± 0.16 $\mu\text{mol/g}$ protein). Hearts perfused with PAG (1 mM) during ischaemia and reperfusion, showed no significant change in the H₂S concentration above control (0.607 ± 0.11 vs. 0.825 ± 0.043 $\mu\text{mol/g}$ protein) (data summarised in figure 5.8).

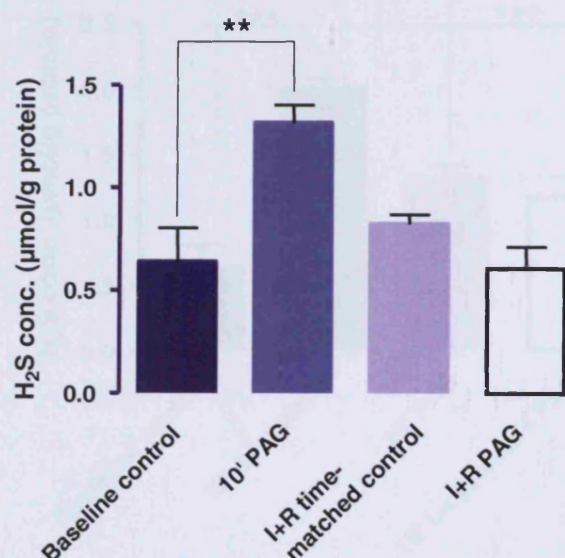


Figure 5.8 Comparison of the H₂S concentration in left ventricle samples from rat isolated heart samples perfused with PAG (1 mM) for either the final 10 minutes of stabilisation, or the final 10 minutes of the 20 minute stabilisation period, or the final 10 minutes of stabilisation plus 35 minutes of regional ischaemia and 10 minutes reperfusion. The time matched controls were perfused with Krebs-Henseleit buffer for the duration of the experiment. The H₂S concentrations of the samples were measured in a biochemical assay, involving release and trapping of the gas. Each value shown is the mean \pm SEM from 5-6 individual hearts. ** $p < 0.01$. (1-way ANOVA).

Measurement of the H₂S concentration in L-cysteine and PAG perfused hearts

Dual perfusion of L-cysteine (1 mM) and PAG (1 mM) caused a highly significant ($p < 0.001$) attenuation of the increased H₂S concentration in the left ventricle seen with L-cysteine alone (1.17 ± 0.15 vs. 2.01 ± 0.069 $\mu\text{mol/g}$ protein), but not below the baseline observed in control (1.17 ± 0.15 vs. 0.639 ± 0.16 $\mu\text{mol/g}$ protein) (data summarised in figure 5.9)

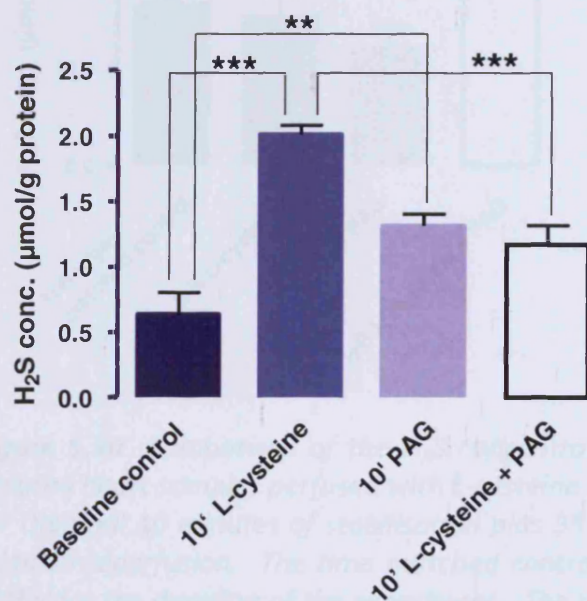


Figure 5.9 Comparison of the H₂S concentration in left ventricle samples from rat isolated heart samples perfused with L-cysteine (1 mM), PAG (1 mM) or in combination for the final 10 minutes of the 20 minute stabilisation period. The time matched controls were perfused with Krebs-Henseleit buffer for the duration of the experiment. The H₂S concentrations of the samples were measured in a biochemical assay, involving release and trapping of the gas. Each value shown is the mean \pm SEM from 5-6 individual hearts. ** $P < 0.01$, *** $p < 0.001$. (1-way ANOVA).

Perfusion with a combination of L-cysteine and PAG during ischaemia and reperfusion did not cause any change in the H_2S concentration compared with baseline (1.10 ± 0.15 vs. $0.825 \pm 0.043 \mu\text{mol/g protein}$ (data summarised in figure 5.10).

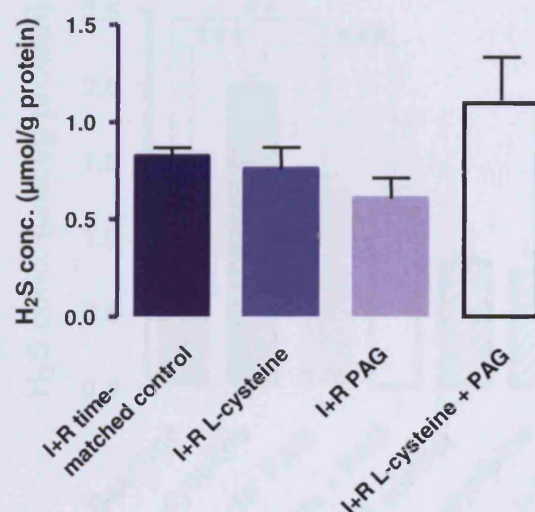


Figure 5.10 Comparison of the H_2S concentration in left ventricle samples from rat isolated heart samples perfused with L-cysteine (1 mM), PAG (1 mM) or in combination for the final 10 minutes of stabilisation plus 35 minutes of regional ischaemia and 10 minutes reperfusion. The time matched controls were perfused with Krebs-Henseleit buffer for the duration of the experiment. The H_2S concentrations of the samples were measured in a biochemical assay, involving release and trapping of the gas. Each value shown is the mean \pm SEM from 5-6 individual hearts. (1-way ANOVA revealed no significant differences).

Summary of the H₂S concentration measurement data

All of the data have been combined in figure 5.11 to enable the significant changes within and between treatment groups to be highlighted.

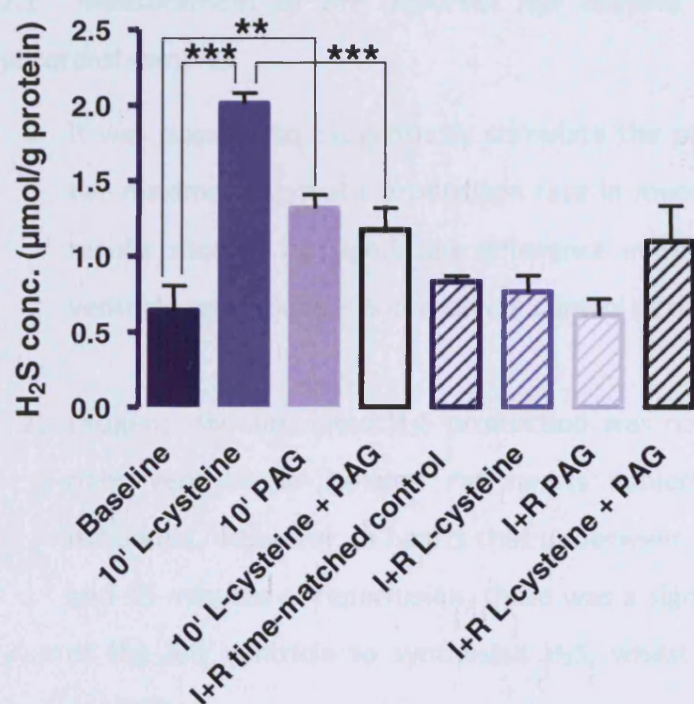


Figure 5.11 Summary of all the H₂S concentrations in left ventricle samples. Comparison of the H₂S concentrations in left ventricle samples from rat isolated Krebs-Henseleit buffer left ventricle samples, either subjected to 20 minutes of stabilisation, or 20 minutes of stabilisation plus 35 minutes of regional ischaemia and 15 minutes reperfusion. The H₂S concentrations of the samples were measured in a biochemical assay, involving release and trapping of the gas. Each value shown is the mean \pm SEM from 5-6 individual hearts. ** $p < 0.01$, *** $p < 0.001$. (1-way ANOVA).

Effects of compounds tested on measured haemodynamic parameters

There was no measurable change in the haemodynamic parameters of the hearts under any of the treatment groups when compared to the control hearts (data not shown).

5.7 Discussion

The findings of the two related studies can be summarised as follows:

5.7.1 Measurement of the maximal H₂S enzyme synthesising capacity of rat myocardial samples

1. It was possible to exogenously stimulate the production of H₂S and measure the maximal enzymatic production rate in myocardium tissue from rats. The results showed no significant difference in the ability of the left and right ventricle to produce H₂S in baseline control perfused hearts.
2. Exogenously stimulated H₂S production was not altered in either the left or right ventricle in isolated rat hearts subjected to 35 minutes regional ischaemia. However, in hearts that underwent 35 minutes regional ischaemia and 15 minutes of reperfusion, there was a significant reduction in the ability of the left ventricle to synthesise H₂S, whilst the right ventricle remained unaffected.

5.7.2 Measurement of the H₂S concentration in rat myocardial samples

1. Left ventricle samples from hearts perfused with Krebs-Henseleit buffer during ischaemia and during ischaemia-reperfusion, showed a significant increase in the basal H₂S concentration compared to baseline control.
2. Left ventricle samples from hearts perfused with Krebs-Henseleit buffer containing L-cysteine for 10 minutes during the stabilisation period, showed a highly significant increase in the H₂S concentration compared to control.
3. This increased H₂S production was attenuated in the presence of the selective CSE inhibitor PAG, but a basal level of H₂S still remained in the left ventricle samples, suggesting an alternative (non)enzymatic source of H₂S.
4. Perfusion with PAG alone during the 20 minute stabilisation caused a significant increase in the H₂S concentration in the left ventricle samples, implicating a compensatory increase in H₂S by an alternative source.

5. Hearts perfused with L-cysteine during ischaemia-reperfusion showed no increase in the H_2S concentration above the time matched control, but both levels were greater than the stabilisation control.
6. Hearts perfused with PAG alone or co-perfusion with L-cysteine did not reduce H_2S levels below time matched control.

A discussion of each series of experiments is given below:

5.7.3 Measurement of the maximal H_2S enzyme synthesising capacity of rat myocardial samples

5.7.3.1 H_2S synthesising ability does not differ in left and right ventricle samples from stabilised hearts and hearts subjected to regional ischaemia

The maximal H_2S synthesising capacity of H_2S in baseline stabilised rat hearts did not differ between the left and right ventricles. These results are not surprising, since there is no reason as to why there should be differences in the maximal H_2S synthesising capacity of the left and right ventricles during normal perfusion. The ischaemic left ventricle's maximal production rate increased 40%, whilst the right ventricle increased 111% compared to control. Neither of these increases were shown to be significantly significant, possibly due to the relatively large error bars. The observation that regional ischaemia had no deleterious effect on the H_2S synthesising ability in either the left or right ventricle, suggests that adequate flow was maintained during ischaemia to preserve the integrity of the enzymes. A possible explanation for the potential of the enzymes to synthesise increased levels of H_2S gas, is that they may have been stimulated to synthesise of H_2S during ischaemia which may have been in preparation for the oxidative stress that occurs at reperfusion.

5.7.3.2 The left ventricle has attenuated H₂S synthesising ability if subjected to ischaemia-reperfusion

However, in hearts subjected to ischaemia and reperfusion, the left ventricle shows a significant 3-fold reduction in H₂S synthesising ability, whilst the right ventricle remains unaffected. Only the flow to the left ventricle would be reduced during regional ischaemia, whilst the flow to the right ventricle would be expected to remain constant since it is not served by the left main anterior coronary artery. These findings suggest that reperfusion may be responsible for the reduced ability of the left ventricle samples to produce H₂S. A possible explanation for this observation is that reperfusion caused an increase in free radicals at reperfusion that may have damaged the enzymes, preventing H₂S production.

It has previously been shown that elevated H₂S levels can exert a negative feedback on the CSE enzymes to reduce the H₂S production rate (Kredich *et al.*, 1973). This implies that if there was a reduction in the left ventricle's ability to synthesise H₂S, such as we observed during ischaemia and reperfusion, then an alternative source of CSE may be stimulated to produce more H₂S in a compensatory fashion. Therefore the right ventricle may have been stimulated to produce more H₂S in an attempt to protect the left ventricle during ischaemia and reperfusion.

5.7.3.3 Relation to previous findings

Only one previous study (Geng *et al.*, 2004c) has investigated the stimulated H₂S production rate of the myocardium (18.64 $\mu\text{mol/g protein/min}$). However, these authors did not specify which region of the myocardium was investigated, but an assumption can be made that it was the left ventricle, since this is the region of interest in regional ischaemia. Since they only investigated the myocardium and no other tissue types, it is difficult to put these rates into context with the H₂S production rates for the myocardium obtained in the present study.

5.7.3.4 Efficiency of the H₂S production assay may be a limiting factor

It is important to note that the efficiency of the H₂S production assay may be limited. Julian *et al.* (2002) implied that the assay may only be about 33% efficient, due to

attempting to detect low concentrations of H₂S and the possibility of the conversion of some H₂S to volatile thiols, despite the anaerobic conditions of the assay. However, in the present investigation it is the relative changes between groups that are important rather than the absolute measurements. This assay has been successfully established after initial preliminary studies and has subsequently been used to obtain reliable and reproducible data.

5.7.3.5 Further studies

Further studies could be conducted, with confidence, to investigate the effects of inhibiting other H₂S synthesising enzymes. Experiments using the CBS inhibitor aminooxyacetic acid and the MST inhibitor 3-mercaptopropionic acid could be used to establish if another enzymatic source of H₂S is responsible for the basal level observed in the myocardium. Also, it would be very interesting to investigate whether there was a restoration of the H₂S synthesising ability in the left ventricle if the period of reperfusion was increased or whether its functionality was permanently impeded.

5.7.4 Measurement of the H₂S concentration in rat myocardial samples

5.7.4.1 Hearts subjected to ischaemia-reperfusion showed increased basal H₂S levels:

The basal concentrations of H₂S were significantly increased ($p < 0.05$) in left ventricle samples from hearts that underwent 20 minutes of stabilisation followed by 35 minutes of regional ischaemia. There was a very significant ($p < 0.01$) increase in the basal tissue concentrations of H₂S in hearts that underwent 20 minutes of stabilisation, 35 minutes of regional ischaemia and 15 minutes of reperfusion. These findings suggest that the endogenous synthesis of H₂S is elevated during ischaemia, and further more during ischaemia-reperfusion perhaps as a protective mechanism by the heart to limit irreversible cell injury. These findings will be discussed later in relation to the L-cysteine perfused hearts.

5.7.4.2 Perfusion with L-cysteine during stabilisation caused increased H₂S synthesis

The H₂S concentration in the left ventricles of hearts perfused with L-cysteine (1 mM) during the 20 minute stabilisation period, or during ischaemia and reperfusion were measured and compared to time matched controls. There was a highly significant

($p < 0.001$) 3-fold increase in the H_2S concentrations in the left ventricle during the 20 minute stabilisation period compared with control. These findings suggest that during the stabilisation period the H_2S synthesising enzymes were viable and capable of exogenous stimulation to produce H_2S . The increased H_2S production was attenuated in the presence of PAG, but not below the basal level of the control group protein. This suggests that an alternative source of H_2S , either enzymatic or non-enzymatic, may be responsible for a basal level of H_2S in the left ventricle in the myocardium. Indeed it is known that there are two other H_2S synthesising enzymes that may potentially exist in the myocardium, CBS and MST (Stipanuk *et al.*, 1982). Although CSE has been reported as the only H_2S synthesising enzyme detected in the myocardium (Ishii *et al.*, 2004), these other enzymes may be weakly expressed and responsible for production of H_2S when CSE is impaired or perhaps responsible for a continuous basal production of H_2S . Further investigation using specific CBS and MST enzyme inhibitors could be used to investigate these enzymes contributions to H_2S production.

5.7.4.3 Perfusion with PAG alone during stabilisation caused an elevation in H_2S concentrations

Interestingly, perfusion with PAG (1mM) during the 20 minute stabilisation period, caused a very significant 2-fold increase in the basal H_2S concentration compared to baseline control, but not as much as L-cysteine (3-fold). A possible explanation for this observation is that due to inhibition of the CSE enzyme with PAG, a compensatory increase in H_2S may be produced by an alternative enzymatic source of H_2S such as CBS or MST, or even a non-enzymatic source which may be responsible for maintaining the basal level in the myocardium.

5.7.4.4 Perfusion with L-cysteine during ischaemia-reperfusion did not increase H_2S synthesis above baseline

L-cysteine is the major sulphur-containing amino acid substrate for CSE and CBS. Perfusion with L-cysteine (1 mM) under aerobic conditions produced a 3-fold increase in H_2S tissue concentration. Perfusion with L-cysteine during ischaemia and reperfusion did not produce a further increase in H_2S levels, compared with its time

matched control or the initial baseline level in the myocardium. This observation may be due to inhibition of the H₂S producing enzymes due to ischaemia and/or reperfusion, preventing a further increase in H₂S production or a lack of ATP to drive active synthesis. This supports the data reported earlier in this study showing a significant impairment in the maximal H₂S synthesising capacity of CSE in left ventricle tissue samples from hearts that underwent ischaemia-reperfusion. It was therefore not a surprising that co-perfusion of the hearts with L-cysteine and PAG during ischaemia and reperfusion caused no significant change in the measured H₂S concentrations compared to time matched controls.

Interestingly it has been shown in chapter 4 that perfusion with L-cysteine during ischaemia and early reperfusion, produced a concentration dependent infarct size limitation, which 1 mM being the most cardioprotective. This reduction in infarct size was attributed to the production of H₂S, which acted as a cardioprotective agent. However, the findings from the studies presented in this chapter suggest that H₂S production may be impaired during ischaemia-reperfusion. This raises the question that perhaps it is not the H₂S gas derived from the donor NaHS, but it is the reactive thiol group, -SH, that acts as a free radical scavenger to induce cardioprotective if the H₂S synthesising enzymes are impaired during ischaemia-reperfusion. Furthermore, the results in this chapter show that perfusion with L-cysteine during baseline stabilisation produced a highly significant increase in the endogenous tissue concentration of H₂S. This supports the idea that L-cysteine can stimulate H₂S production but only under aerobic conditions and therefore during ischaemia-reperfusion L-cysteine may be acting as a free radical scavenger. In addition these findings provide further evidence of the existence of H₂S synthesising enzymes in the rat myocardium, despite the limited data generated from the Western blotting studies in chapter 3.

5.7.4.5 CSE enzyme in the right ventricle as a potential source of H₂S for left ventricle

The right ventricle is a potential alternative source of H₂S for the left ventricle since it is unlikely to have been effected by regional ischaemia as adequate flow was maintained thus preserving the CSE enzymes. However, since it was not possible to measure the tissue concentrations of H₂S from right ventricle samples in this study, it is difficult to prove its involvement. If indeed the right ventricle was supplying the left with H₂S during ischaemia-reperfusion, then the levels of H₂S observed in the left ventricle would be expected to be elevated above the time matched control. This is because stimulated H₂S synthesis would be expected to occur in the right ventricle of hearts perfused with L-cysteine. Since this does not appear to have occurred, it suggests an alternative CSE independent source of H₂S. Interestingly perfusion with PAG during ischaemia-reperfusion did not effect the basal levels of H₂S, suggesting a CSE independent source because even if the CSE enzymes in the right ventricle remained unaffected by ischaemia-reperfusion, the presence of PAG would prevent them supplying additional H₂S. Therefore it appears a CSE independent source of H₂S must be present.

5.7.4.6 Reasoning for elevated H₂S levels during ischaemia-reperfusion despite impaired CSE function

Interestingly the significantly elevated H₂S concentrations observed in left ventricle samples from Krebs-Henseleit buffer perfused hearts subjected to ischaemia and ischaemia-reperfusion were not observed to the same degree in hearts subjected to the same perfusion protocol but with the addition of L-cysteine perfusion for the duration of ischaemia and first 10 minutes of reperfusion. There was a modest, but not statistically significant, increase in the concentration of H₂S measured in the left ventricle of L-cysteine perfused hearts and their time matched Krebs-Henseleit buffer perfused controls. The reasoning behind the lack of significantly increased H₂S production in the L-cysteine perfused hearts is difficult to explain, since they only differ by the addition of the L-cysteine to the Krebs-Henseleit buffer solution. A simple explanation is that the baseline control group of the L-cysteine perfused hearts contained a higher concentration of H₂S than the Krebs-Henseleit buffer perfused

hearts to start with and had a relatively large error and therefore the small increases observed during ischaemia-reperfusion are not statistically significant.

The observation that there was no further increase in the H₂S concentration in the L-cysteine perfused hearts during ischaemia-reperfusion, compared to the time matched control, suggests that stimulating the CSE enzyme with substrate and co-factor produces no additional H₂S, and an alternative source of H₂S may exist to provide the elevated basal H₂S levels observed in the left ventricle. Therefore the myocardium may utilise an alternative non-enzymatic source of H₂S during ischaemia-reperfusion. This seems feasible because during ischaemia-reperfusion enzymes are likely to become impaired due to factors such as lack of ATP, toxic metabolic accumulation, and free radical generation. Therefore it appears that there may be a more robust source of H₂S production to maintain basal levels which may even act in a possible attempt to elevate levels to induce endogenous cardioprotection.

5.7.4.7 The potential role of a non-enzymatic source of H₂S production

A non-enzymatic source of H₂S also exists where elemental sulphur is reduced using reducing equivalents obtained from the oxidation of glucose (Searcy *et al.*, 1998). All of the essential components of this pathway are present *in vivo* including the supply of sulphur. This may therefore be an alternative source of H₂S production when the enzymes are not viable such as during ischaemia and reperfusion. This source of H₂S may also have been contributing to the basal H₂S levels observed in these experiments. A specific inhibitor of this pathway would enable its role to be further investigated.

5.7.4.8 Efficiency of the H₂S production assay may be a limiting factor

This assay is also of a similar nature to the stimulated H₂S production assay and as a result may suffer from the same issues regarding efficiency. Again, however this is not of great importance, since relative changes are of interest.

5.7.4.9 Further studies

It would have been interesting to measure the tissue concentration of H₂S in the right ventricle and see how they related to the enzyme activity. Unfortunately this assay required reasonably large quantities of tissues, thus preventing the right ventricle being used. A possible option would be to miniaturise the assay, but this may result in a loss of sensitivity. Further studies could be performed with the left ventricle and other specific inhibitors of H₂S synthesising enzymes to gain a further understanding of the other (non)enzymatic sources of H₂S.

In addition, measurement of the exogenously stimulated enzyme derived H₂S and tissue concentrations of H₂S, could have been measured using an H₂S probe. These are known to exist for use in environmental monitoring of H₂S. This would have replaced the need to trap the gas on filter paper and removal several stages from the labour intensive assay. However, it appears that the probes available would not be sensitive enough for the detection of the relatively low concentrations of H₂S generated. Interestingly a paper by Doeller *et al.* (2005) describes the use of a novel polarographic probe H₂S sensor with a high signal to noise ratio and signal specificity to H₂S. It is highly sensitive and can detect H₂S concentrations as low as 10 nM and can also be used to measure the consumption of H₂S by mammalian tissues. It will be interesting to see if this gains acceptance for future H₂S measurement studies.

5.8 Conclusion

The findings from these studies suggest that left and right ventricle tissue samples obtained from the rat myocardium are able to synthesise H_2S when the enzymes are stimulated with the substrate L-cysteine and co-factor pyridoxal-5-phosphate, which can be collected and measured. It is also possible to release and measure the concentration of H_2S contained within the tissue. The results from this study show that H_2S concentrations were elevated in hearts that underwent ischaemia-reperfusion, suggesting that endogenous H_2S levels may be elevated in an attempt to protect the myocardium from ischaemia-reperfusion injury. However, the ability of the left ventricle to synthesise H_2S after ischaemia-reperfusion was impaired, whilst the right ventricle remained unaffected, suggesting a role of the right ventricle to supply H_2S to the left ventricle. A robust non-enzymatic H_2S source may also exist which contributes to the basal level observed in the heart and may even supply H_2S during ischaemia-reperfusion. Therefore there appears to be a complex interplay between various enzymatic and non-enzymatic sources of H_2S in the myocardium, only some of which this study has explored. These may act to serve as a protective mechanism to ensure that H_2S is always present at a basal level in the myocardium and may compensate for reduced enzyme activity during ischaemia-reperfusion. The findings from this study also act to verify the PCR and Western blotting results, reported in Chapter 3, that CSE and potentially CBS H_2S synthesising enzymes are present in the rat myocardium.

Chapter 6 Investigation into the role of H₂S in activation of the Reperfusion Salvage Kinase (RISK) pathway, as a potential mechanism of cardioprotection

6.1 Introduction

Studies reported in earlier chapters of this thesis gradually build upon the existing knowledge of H₂S as a physiological gaseous mediator and attempt to expand the knowledge of its existence and role within in the myocardium. In particular it has been confirmed that the myocardium possesses the ability to enzymatically generate H₂S, and that stimulated H₂S production was cardioprotective. However, the mechanism of the cardioprotective action of H₂S has yet to receive attention. The series of experiments conducted in this chapter aimed to decipher the cardioprotective mechanism of action of NaHS. In addition thiol containing compounds with more structural complexity than NaHS were investigated which may give a greater insight into the mechanism of action of NaHS.

6.1.1 H₂S as a potential activator of the RISK pathway

The RISK pathway has already been comprehensively described in Chapter 1. However a brief overview of the RISK pathway in context of the potential activation by NaHS and subsequent cardioprotection will now be discussed. It has been shown that a number of factors activate the RISK pathway to cause cardioprotection (Hausenloy *et al.*, 2007). These mediators act through a variety of mechanisms including G-protein coupled receptors, growth factor receptors and naturetic peptide receptors (Hausenloy *et al.*, 2007). It has also been shown that volatile anaesthetics can act via non-receptor mediated pathways (Chiari *et al.*, 2005). This vast variety of activators and mechanisms suggests that there is great potential for many undiscovered activators of the RISK pathway, particularly through a non-receptor mediated mechanism, to induce cardioprotection. Indeed there is much evidence to support the assumption that H₂S may induce cardioprotection via action of the RISK pathway. This is based on the observations that H₂S limits infarct size when hearts are perfused during ischaemia and early reperfusion and its mechanism of cardioprotection is K_{ATP} channel dependent (Johansen *et al.*, 2006), which is an important component of the RISK pathway (Gross *et al.*, 1999; Gross *et al.*, 2003). In addition H₂S and NO are known to interact (Li *et al.*, 2005; Mok *et al.*, 2004; Zhao *et al.*, 2002), and since NO is a fundamental mediator of the RISK pathway (Dimmeler *et al.*, 1999), it suggests that

H₂S may have a role in mediating its actions. Taking these findings together with the potential to activate the RISK pathway through a non-receptor mediated fashion, since no pharmacological target of H₂S had been discovered, a series of experiments were proposed to investigate whether H₂S activates the RISK pathway to induce cardioprotection.

6.1.2 MESNA

The H₂S donor NaHS has been selected for use throughout the studies involving H₂S generation in this thesis. This is because there are very few alternatives and is widely used by many other research groups making it possible to directly compare results between investigators (Bian *et al.*, 2006; Elrod *et al.*, 2007; Hu *et al.*, 2008; Ji *et al.*, 2008; Johansen *et al.*, 2006; Pan *et al.*, 2006; Sivarajah *et al.*, 2006; Sodha *et al.*, 2008; Zhang *et al.*, 2007; Zhu *et al.*, 2007). It is also the simplest thiol containing compound other than H₂S gas. Since thiols are known to act as free radical scavengers, it is conceivable that the thiol component of NaHS may acts to induce cardioprotection, as opposed to the generation of H₂S gas. To investigate this concept further a more complex thiol containing compound, 2-mercaptoethane sulphonate sodium (MESNA), was also selected, enabling the cardioprotective effect of two potential H₂S donors, but structurally different thiols, to be compared (see figure 6.1 for structure of MESNA). Prior to conducting these studies there was to be no other published work investigating the potential cardioprotective effects of MESNA.

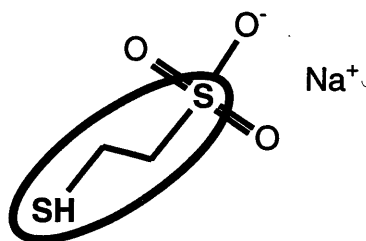


Figure 6.1 Structure of MESNA with the thiol group highlighted. The structure of MESNA is far more complex than that of NaHS.

MESNA is primarily used in chemotherapy regimens for the prevention of haemorrhagic cystitis induced by the oxazaphosphorines cyclophosphamide and

ifosfamide (Dechant *et al.*, 1991). Aside from MESNA's urothelial protective ability, it has also been shown to protect the intestinal mucosa by reducing the toxicity of cisplatin (Allan *et al.*, 1986) and ifosfamide (Ypsilantis *et al.*, 2004) or reducing intestinal inflammation in experimental colitis (Shusterman *et al.*, 2003). Other published studies have investigated the protective effects of MESNA in ischaemia-reperfusion in the liver, kidney, and intestine (Ypsilantis *et al.*, 2006). MESNA's thiol group is thought to be responsible for its antioxidant property which enables it to act as a free radical scavenger and thus limiting the damage associated with ischaemia-reperfusion when oxidant or ROS levels are significantly increased (Gressier *et al.*, 1994). MESNA is also a small molecule which enables it to concentrate in the kidneys and therefore increase its ability as an anti-oxidant free radical scavenger (Gressier *et al.*, 1994).

6.2 Aim

The aim of this series of experiments was to investigate if H₂S activated the reperfusion injury salvage kinase (RISK) pathway, as a potential mechanism of cardioprotection. The cardioprotective action of the structurally dissimilar and more complex thiol-containing compound MESNA was also investigated as an alternative to NaHS in relation to activation of the RISK pathway.

6.3 Hypotheses

1. H₂S protection against ischaemia-reperfusion injury in the isolated rat perfused Langendorff heart by activation of the RISK pathway
2. The thiol-containing compound MESNA will induce cardioprotection via activation of the RISK pathway

6.4 Objectives

These hypotheses were tested through the following experimental objectives:

1. To determine when H₂S exerts its protective action, by perfusing hearts with Krebs-Henseleit buffer containing the H₂S donor NaHS at three different time points: pre-ischaemically with wash-out (pharmacological preconditioning); during ischaemia; and at reperfusion.
2. To investigate if H₂S activates the RISK pathway by the use of pharmacological inhibitors.
3. To determine if MESNA is cardioprotection using two different treatment protocols: pharmacological preconditioning with wash-out, and at reperfusion.
4. To investigate if MESNA activates the RISK pathway by the use of pharmacological inhibitors.

6.5 Methods

6.5.1 Source of compounds used

The H₂S donor NaHS was purchased from Acros Organics, UK. The PI3K inhibitor (LY294002), the Akt inhibitor (Akti VIII), and the thiol containing compound 2-mercaptoethane sulphonate sodium (MESNA), were all obtained purchased from Sigma-Aldrich, UK. LY294002 and Akti VIII were dissolved in DMSO, and required vehicle controls (DMSO 0.05%), whilst NaHS and MESNA were dissolved in Krebs-Henseleit buffer and therefore required no vehicle controls.

6.5.2 Preparation of Langendorff perfused isolated rat hearts

6.5.2.1 Setup of isolated heart perfusion

Rat isolated hearts were used to investigate the role of NaHS and MESNA in cardioprotection. The hearts were Langendorff perfused using the methods described in Chapter 2, section 2.2. All hearts received an initial 10 minute stabilisation period following cannulation, to establish sinus rhythm. The hearts were then randomly assigned to one of the groups within each study described below:

6.5.3 NaHS perfusion protocols

6.5.3.1 Study 1: Perfusion of hearts with NaHS during early reperfusion

Treatment protocols for this study are illustrated in Figure 6.2.

Group 1 – Control: After an initial 20 minutes of stabilisation, hearts were subjected to 35 minutes regional ischaemia, and a 120 minute reperfusion period. For the duration of the experiment hearts were perfused with Krebs-Henseleit buffer.

Groups 2 to 4 – NaHS 1, 10 or 50 μ M early reperfusion: After 20 minutes of stabilisation, and 30 minutes of ischaemia hearts were perfused with Krebs-Henseleit buffer containing NaHS 1 μ M (group 2), 10 μ M (group 3) or 50 μ M (group 4) for the remaining 5 minutes of regional ischaemia until the first 10 minutes of reperfusion.

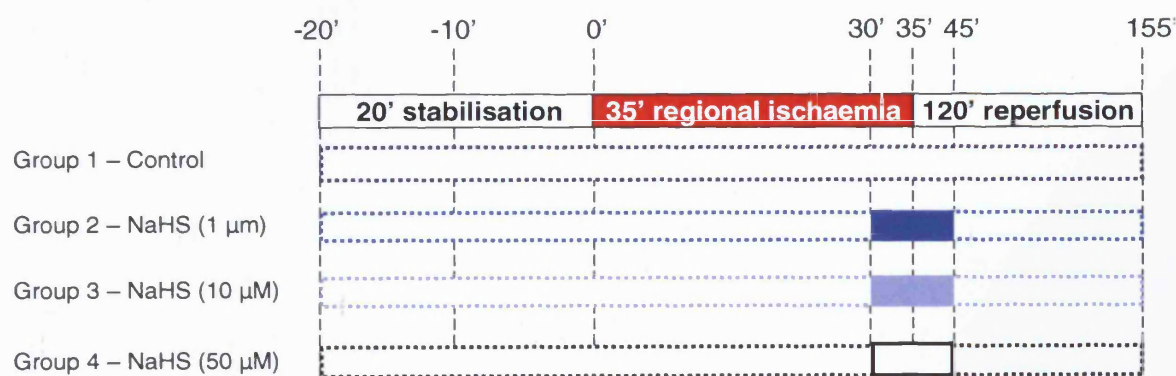


Figure 6.2 Summary of the perfusion protocol for Study 1: Perfusion of hearts with NaHS at early reperfusion. For the final 5 minutes of regional ischaemia and the initial 10 minutes of reperfusion, the treatment hearts were perfused with NaHS 1-50 μ M) and then switched back to Krebs-Henseleit buffer for the duration of the reperfusion period.

6.5.3.2 Study 2: Perfusion of hearts with NaHS during early ischaemia

Treatment protocols for this study are illustrated in Figure 6.3.

Group 1 – Control: After an initial 20 minutes of stabilisation, hearts were subjected to 35 minutes regional ischaemia, and a 120 minute reperfusion period. For the duration of the experiment hearts were perfused with Krebs-Henseleit buffer.

Groups 2 to 3 – NaHS 1, or 10 μ M early ischaemia: After 10 minutes of stabilisation, hearts were perfused with Krebs-Henseleit buffer containing NaHS 1 μ M (group 2) or 10 μ M (group 3) for the remaining 10 minutes of stabilisation and the first 10 minutes of regional ischaemia until the first 10 minutes of regional ischaemia.

Group 4 – NaHS 1 μ M duration of ischaemia and early reperfusion: As per group 2 except that hearts were perfused with Krebs-Henseleit buffer containing 1 μ M NaHS for the duration of regional ischaemia until the first 10 minutes of reperfusion.

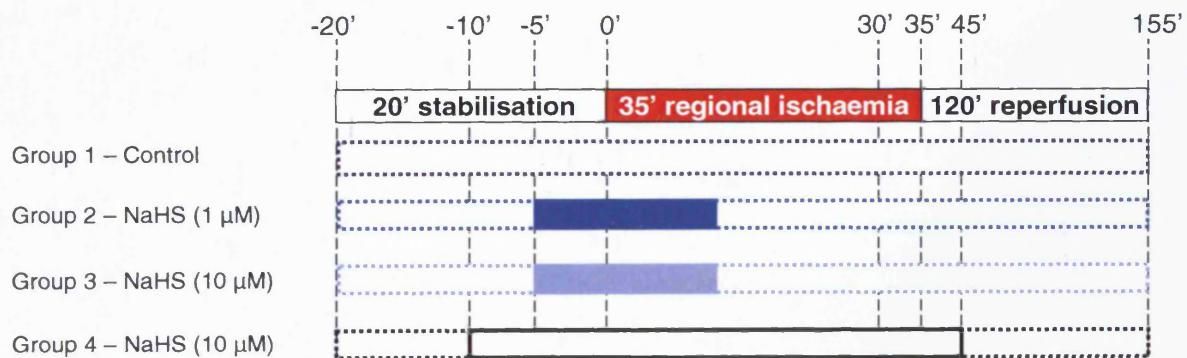


Figure 6.3 Summary of the perfusion protocol for Study 2: Perfusion of hearts with NaHS during ischaemia. For the final 5 minutes of stabilisation and the initial 10 minutes of regional ischaemia, the treatment hearts were perfused with NaHS 1-10 μ M) and then switched back to Krebs-Henseleit buffer for the duration of the reperfusion period. Group 4 hearts which were perfused with NaHS (1 μ M) after 10 minutes of stabilisation and for the duration of ischaemia and until the initial 10 minutes of reperfusion.

6.5.3.3 Study 3: Pharmacological preconditioning of hearts with NaHS

Treatment protocols for this study are illustrated in Figure 6.4.

Group 1 – Control: After an initial 20 minutes of stabilisation, hearts were subjected to 35 minutes regional ischaemia, and a 120 minute reperfusion period. For the duration of the experiment hearts were perfused with Krebs-Henseleit buffer.

Group 2 – 1 x 5' NaHS 50 μ M: After 10 minutes of stabilisation, hearts were perfused with Krebs-Henseleit buffer containing NaHS 50 μ M for 5 minutes before returning to Krebs-Henseleit buffer perfusion for the remaining 5 minutes of stabilisation and duration of experiment.

Group 3 – Global ischaemic preconditioning: After 10 minutes of stabilisation, total flow to the hearts was stopped for 5 minutes and hearts were submerged in a heart jacket containing warmed Krebs-Henseleit buffer to maintain temperature. After 5 minutes the hearts were reperfused for 5 minutes before inducing regional ischaemia.

Group 4 – 2 x 5' NaHS 50 μ M: After 10 minutes of stabilisation, hearts were perfused with Krebs-Henseleit buffer containing NaHS 50 μ M for 5 minutes, then perfused with Krebs-Henseleit buffer for 5 minutes. This cycle of 5 minutes NaHS followed by 5 minutes Krebs-Henseleit buffer was repeated again prior to inducing regional ischaemia.

Group 5 – 2 x 5' global ischaemia: After 10 minutes of stabilisation, total flow to the hearts was stopped for 5 minutes and hearts were submerged in a heart jacket containing warmed Krebs-Henseleit buffer to maintain temperature. After 5 minutes the hearts were reperfused for 5 minutes. This cycle of 5 minutes regional ischaemia followed by 5 minutes reperfusion was repeated again prior to inducing regional ischaemia.

Group 6 – 3 x 3' NaHS 50 μ M: After 10 minutes of stabilisation, hearts were perfused with Krebs-Henseleit buffer containing NaHS 50 μ M for 3 minutes, then perfused with Krebs-Henseleit buffer for 5 minutes. This cycle of 3 minutes NaHS followed by 5

minutes Krebs-Henseleit buffer was repeated twice prior to inducing regional ischaemia.

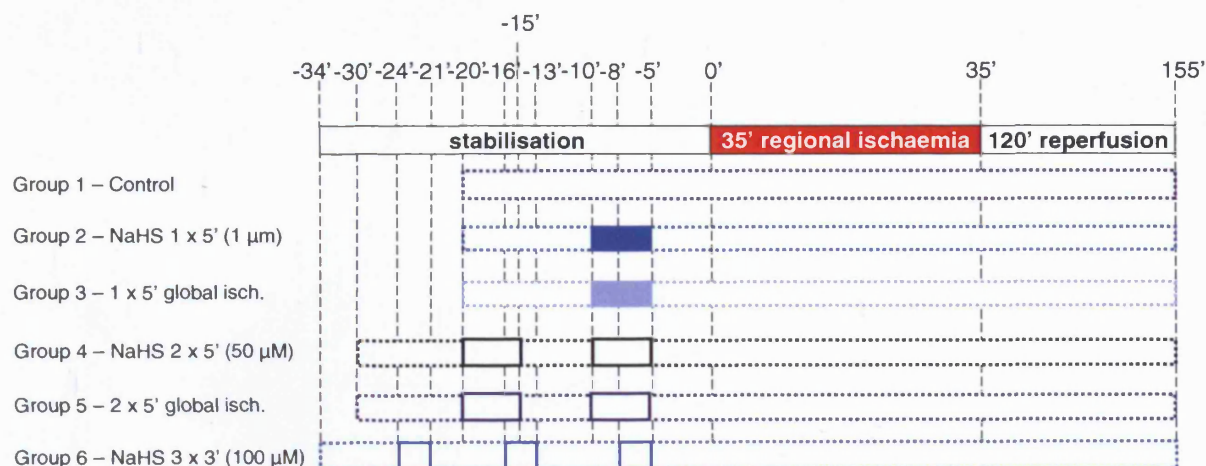


Figure 6.4 Summary of the perfusion protocol for Study 3: Pharmacological preconditioning of hearts with NaHS. Hearts were pharmacologically preconditioned with NaHS using various protocols. Time matched ischaemic preconditioning controls were also performed.

Once it had been established that preconditioning with NaHS 50 μ M was the most effective method of inducing cardioprotection in isolated rat hearts, a second series of experiments were performed using inhibitors of the RISK pathway in order to determine whether the cardioprotection observed with NaHS was via activation of the RISK pathway.

6.5.3.4 Study 4: Effect of PI3K and Akt inhibition on NaHS preconditioning

Treatment protocols for this study are illustrated in Figure 6.5.

Group 1 – Control: After an initial 20 minutes of stabilisation, hearts were subjected to 35 minutes regional ischaemia, and a 120 minute reperfusion period. For the duration of the experiment hearts were perfused with Krebs-Henseleit buffer. To confirm that the DMSO vehicle used for the inhibitors had no effect on the hearts, hearts ($n=3$) were perfused with Krebs-Henseleit buffer containing DMSO (0.05%) for the remaining 5 minutes of ischaemia and initial 10 minutes of reperfusion.

Group 2 – NaHS 50 μ M: After 15 minutes of stabilisation, hearts were perfused with Krebs-Henseleit buffer containing NaHS 50 μ M for the remaining 5 minutes of stabilisation and the first 10 minutes of regional ischaemia

Group 3 – NaHS 50 μ M + LY294002 10 μ M: As per group 2, with the addition that hearts were perfused with Krebs-Henseleit buffer containing LY294002 10 μ M for the final 5 minutes of regional ischaemia and first 10 minutes of reperfusion.

Group 4 – LY294002 10 μ M: For the final 5 minutes of regional ischaemia and first 10 minutes of reperfusion hearts were perfused with Krebs-Henseleit buffer containing LY294002 10 μ M.

Group 5 – NaHS 50 μ M + Akti VIII 2 μ M: As per group 2, with the addition that hearts were perfused with Krebs-Henseleit buffer containing Akti VIII 2 μ M for the final 5 minutes of regional ischaemia and first 10 minutes of reperfusion.

Group 6 – Akti VIII 2 μ M: As per group 4, except hearts were perfused with Krebs-Henseleit buffer containing Akti VIII 2 μ M.

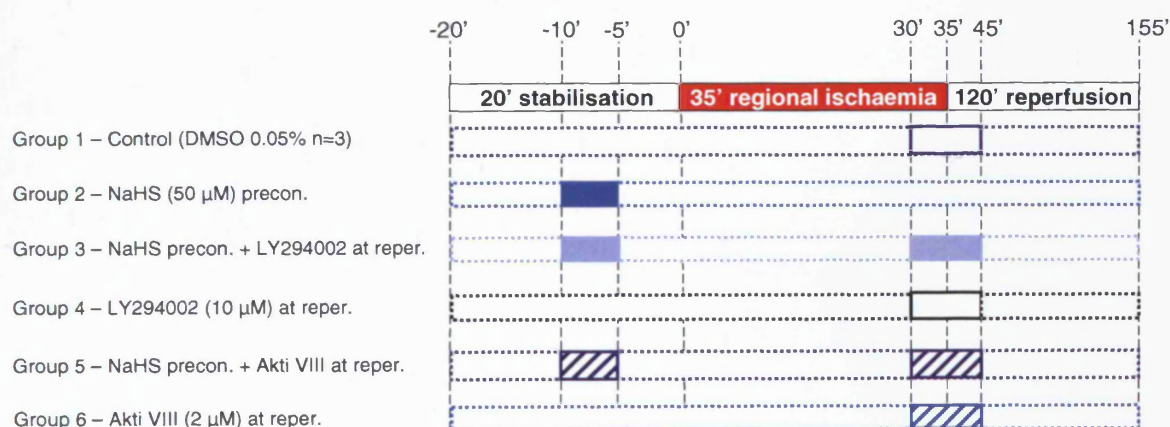


Figure 6.5 Summary of the perfusion protocol for Study 4: Effect of inhibition of PI3K on NaHS preconditioning. After 10 minutes of stabilisation hearts were preconditioned with NaHS for 5 minutes and then back to Krebs-Henseleit buffer for the final 5 minutes of stabilisation. After NaHS preconditioning, hearts were perfused with the PI3K inhibitor, LY294002, or Akt inhibitor, Akti VIII, at early reperfusion.

6.5.4 MESNA perfusion protocols

6.5.4.1 Study 5: Pharmacological pre- and postconditioning of hearts with MESNA

Treatment protocols for this study are illustrated in Figure 6.6.

Group 1 – Control: After an initial 20 minutes of stabilisation, hearts were subjected to 35 minutes regional ischaemia, and a 120 minute reperfusion period. For the duration of the experiment hearts were perfused with Krebs-Henseleit buffer.

Group 2 – 50 μ M MESNA preconditioning: After 15 minutes of stabilisation, hearts were perfused with Krebs-Henseleit buffer containing MESNA 50 μ M for the remaining 5 minutes of stabilisation and the first 10 minutes of regional ischaemia.

Group 3 – 50 μ M MESNA at early reperfusion: After 20 minutes of stabilisation, and 30 minutes of regional ischaemia hearts were perfused with Krebs-Henseleit buffer containing MESNA 50 μ M for the remaining 5 minutes of regional ischaemia until the first 10 minutes of reperfusion.

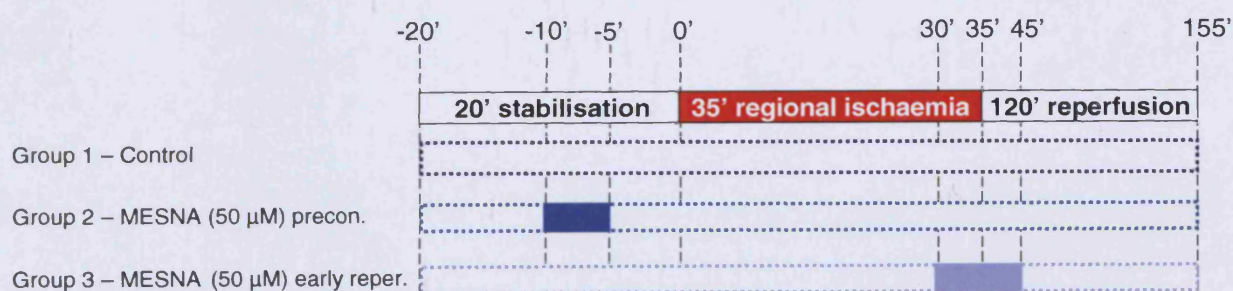


Figure 6.6 Summary of the perfusion protocol for Study 5: Pharmacological pre- and postconditioning of hearts with MESNA. Hearts were either preconditioned with MESNA for 5 minutes during the stabilisation period or postconditioned with MESNA for the final 5 minutes of regional ischaemia and first 10 minutes of reperfusion.

6.5.4.2 Study 6: Effect of PI3K inhibition on cardioprotection induced by MESNA at early reperfusion

Treatment protocols for this study are illustrated in Figure 6.7.

Group 1 – Control: After an initial 20 minutes of stabilisation, hearts were subjected to 35 minutes regional ischaemia, and a 120 minute reperfusion period. For the duration of the experiment hearts were perfused with Krebs-Henseleit buffer. To confirm that the DMSO vehicle used for the inhibitor had no effect on the hearts, hearts (n=4) were perfused with Krebs-Henseleit buffer containing DMSO (0.05%) for the remaining 5 minutes of ischaemia and initial 10 minutes of reperfusion.

Group 2 – 50 μ M MESNA at early reperfusion: After 20 minutes of stabilisation, and 30 minutes of regional ischaemia hearts were perfused with Krebs-Henseleit buffer containing MESNA 50 μ M for the remaining 5 minutes of regional ischaemia until the first 10 minutes of reperfusion.

Group 3 – MESNA at early reperfusion + LY294002: As per group 2, except that hearts were perfused with Krebs-Henseleit buffer containing MESNA 50 μ M and LY294002 50 μ M.

Group 4 – LY294002 10 μ M: As per group 2, except hearts were perfused with Krebs-Henseleit buffer containing LY294002 10 μ M.

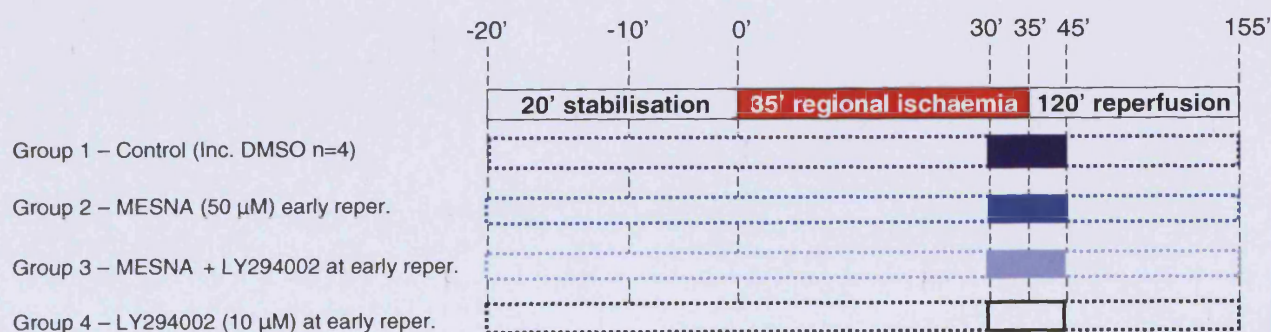


Figure 6.7 Summary of the perfusion protocol for Study 6: Effect of PI3K inhibition on cardioprotection induced by MESNA at early reperfusion. For the final 5 minutes of ischaemia and initial 10 minutes of reperfusion, hearts were perfused with Krebs-Henseleit buffer containing MESNA. Hearts were also perfused with MESNA and the PI3linase inhibitor LY294002 at using the same protocol.

6.6 Results

6.6.1 Exclusion criteria

In total 168 rat hearts were used for the experiments performed in these studies. Of these 168 hearts, 17 were excluded on technical grounds. Data from 151 rat hearts are presented in this chapter.

6.6.2 NaHS perfusion data

6.6.2.1 Study 1: Perfusion of hearts with NaHS at early reperfusion

Perfusing the rat heart with NaHS (1-50 μ M) at early reperfusion had no significant effect at reducing the infarct size ($37.7 \pm 4.6\%$, $29.2 \pm 3.8\%$ and $35.7 \pm 7.7\%$ vs. $39.6 \pm 3.3\%$ for 1 μ M, 10 μ M, 50 μ M and control respectively). There was a slight tendency of the 10 μ M concentration to reduce the infarct size ($29.2 \pm 3.8\%$ vs. $39.6 \pm 3.3\%$ for 10 μ M NaHS and control respectively), but this was not significant (as shown in figure 6.8).

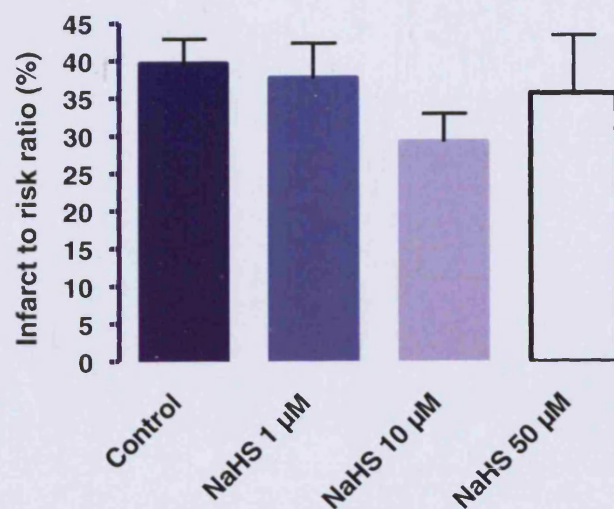


Figure 6.8 Comparison of infarct to risk ratios (%) for concentration ranging studies with the H_2S donor NaHS (1-50 μ M) at early reperfusion. NaHS had no significant effect at reducing infarct size at any of the concentrations tested. One-way ANOVA was used to test for significance differences between all groups. Each value shown is the mean \pm SEM from 6-13 individual heartss.

6.6.2.2 Study 2: Perfusion of hearts with NaHS during early ischaemia

Perfusing the rat heart with NaHS (1-10 μM) during early ischaemia had no significant effect at reducing the infarct size ($36.9 \pm 4.7\%$ and $30.3 \pm 2.6\%$ vs. $39.6 \pm 3.3\%$ for 1 μM , 10 μM and control respectively). There was a slight tendency of the 10 μM concentration to reduce the infarct size ($30.3 \pm 2.6\%$ vs $39.6 \pm 3.3\%$ for 10 μM NaHS and control respectively), as was seen with NaHS at reperfusion, but this was not significant. Perfusing hearts with NaHS 1 μM for the duration of ischaemia and early reperfusion, produced no reduction in infarct size compared to control ($33.4 \pm 3.5\%$ vs. $39.6 \pm 3.3\%$) (data summarised in figure 6.9).

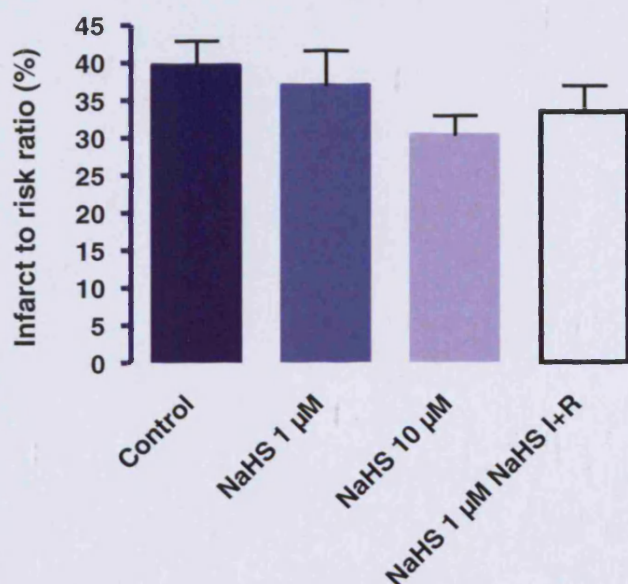


Figure 6.9 Comparison of infarct to risk ratios (%), for concentration ranging studies with the H_2S donor NaHS (1 - 10 μM) at early ischaemia and also perfusion with NaHS (1 μM) for the duration of ischaemia and early reperfusion. NaHS produced no significant effect at reducing infarct size at any of the concentrations tested. One-way ANOVA was used to test for significance differences between all groups. Each value shown is the mean \pm SEM from 4-13 individual hearts.

6.6.2.3 Coronary flow and contractility data for studies 1 and 2

Baseline cardiodynamic data (heart rate, coronary flow rate, developed pressure and rate pressure product for the hearts in studies 1 and 2 are summarised in figure 6.10. The rate pressure product and coronary flow rate of the hearts throughout the two experiments is presented in figure 6.11. There were no significant effects of any of the compounds tested on the measured parameters. Regional ischaemia caused a

reduction in rate pressure product and coronary flow rate in all treatment groups as expected (as shown in figure 6.11). Run-down of the hearts is demonstrated by a reduction in rate pressure product in all treatment groups as expected (as shown in figure 6.11).

Treatment group	n	CFR ml/min	HR BPM	LVDP mmHg	RPP mmHg/min x10 ³	RV and LV vol. cm ³	Risk zone vol. cm ³
Control	15	17.7 ± 0.9	296 ± 16	112.3 ± 7.5	32.6 ± 2.1	0.84 ± 0.02	0.40 ± 0.03
NaHS 1 µM early isch.	4	15.8 ± 0.7	198 ± 26	86.6 ± 7.3	26.3 ± 3.6	0.94 ± 0.09	0.45 ± 0.05
NaHS 10 µM early isch.	4	17.9 ± 1.0	311 ± 19	109.7 ± 7.9	34.4 ± 3.4	0.63 ± 0.04	0.36 ± 0.02
NaHS 50 µM isch. and early reper	9	17.2 ± 1.2	248 ± 16	106.1 ± 7.0	26.1 ± 1.8	0.84 ± 0.02	0.39 ± 0.02
NaHS 1 µM early reper.	6	16.2 ± 2.2	297 ± 17	96.8 ± 10.6	28.1 ± 2.0	0.76 ± 0.03	0.37 ± 0.03
NaHS 10 µM early reper.	8	18.0 ± 1.2	300 ± 35	108.9 ± 2.9	32.8 ± 4.3	0.85 ± 0.02	0.46 ± 0.04
NaHS 50 µM early reper.	6	19.5 ± 1.4	332 ± 14	94.2 ± 4.2	31.4 ± 2.0	0.82 ± 0.02	0.44 ± 0.03

Figure 6.10 *Baseline cardiodynamic data for hearts used in the NaHS early ischaemia and early reperfusion perfusion studies. Abbreviations: LV = left ventricle, RV = right ventricle, CFR = coronary flow rat, HR = heart rate, LVDP = left ventricular pressure, RPP = rate pressure product (HR x LVDP).*

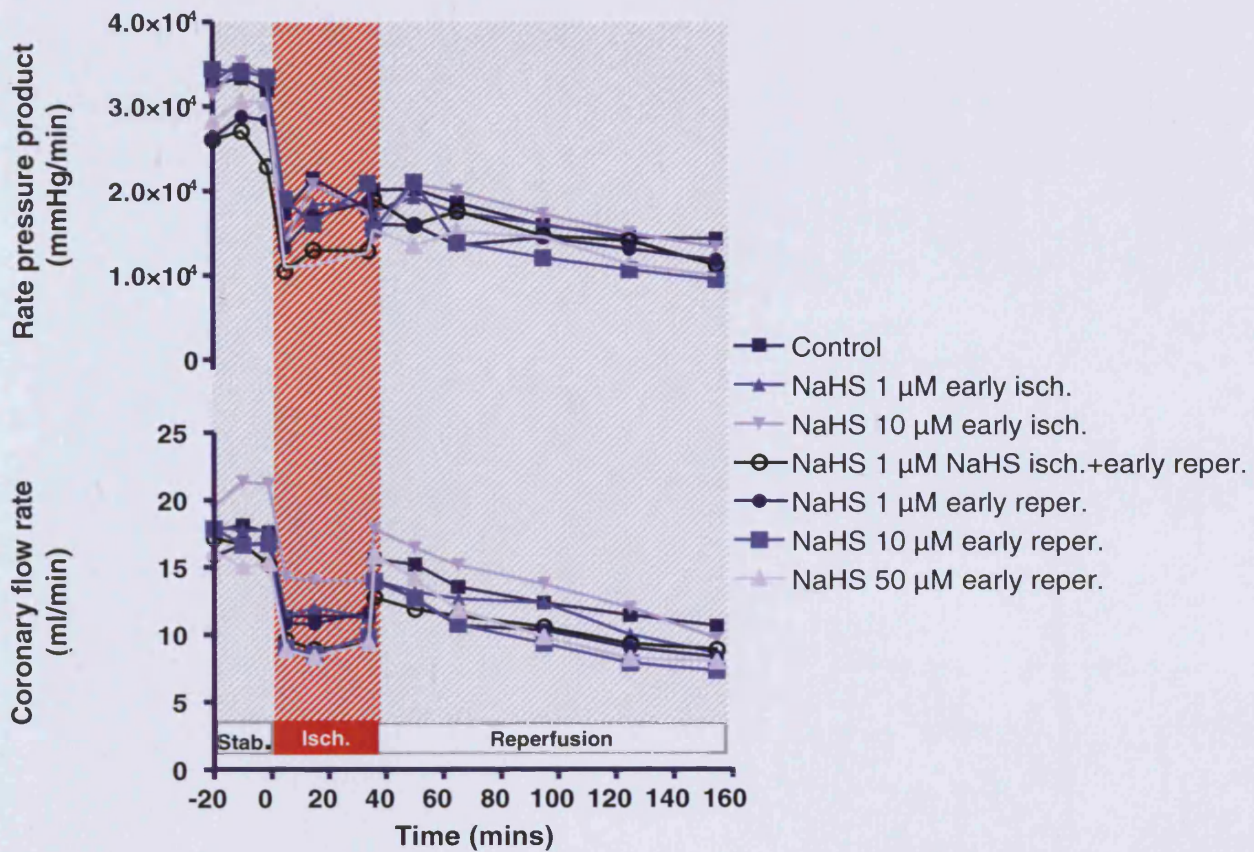


Figure 6.11 Rate pressure product (RPP) (A) and coronary flow rate (CFR) (B) for hearts used in the NaHS early ischaemia and early reperfusion perfusion studies. Stab.=stabilisation period (20 mins), Isch.=Regional ischaemia (35 mins), Reperfusion (120 mins). Each value shown is the mean from 4-15 individual hearts. SEM error bars have been removed for clarity.

6.6.2.4 Study 3: Pharmacological preconditioning of hearts with NaHS

A single preconditioning stimulus of NaHS (50 μ M) prior to a prolonged ischaemia–reperfusion episode induced a very significant reduction in the infarct size compared to control ($27.7 \pm 2.1\%$ vs. $39.6 \pm 3.3\%$, $**p < 0.01$). This was comparable to the single 5 minute episode of global ischaemic preconditioning (Glsch.) ($28.6 \pm 2.6\%$) which acted as a positive control. Hearts perfused with a dual preconditioning stimulus (2 x 5 minutes) of NaHS (50 μ M) did not induce cardioprotection and produced an increase in infarct size in comparison to control ($45.8 \pm 3.3\%$ vs. $39.6 \pm 3.3\%$) although this was not significant. This was in contrast to the two episodes of 5 minute global ischaemia which caused a significant reduction in infarct size in comparison to control ($26.1 \pm 3.3\%$ vs. $39.6 \pm 3.3\%$, $*p < 0.05$). Increasing the preconditioning stimulus of NaHS to three, 3 minute bursts of NaHS, caused no significant reduction in infarct size in comparison to control ($33.3 \pm 1.1\%$ vs. $38.0 \pm 3.5\%$) (data summarised in figure 6.12).

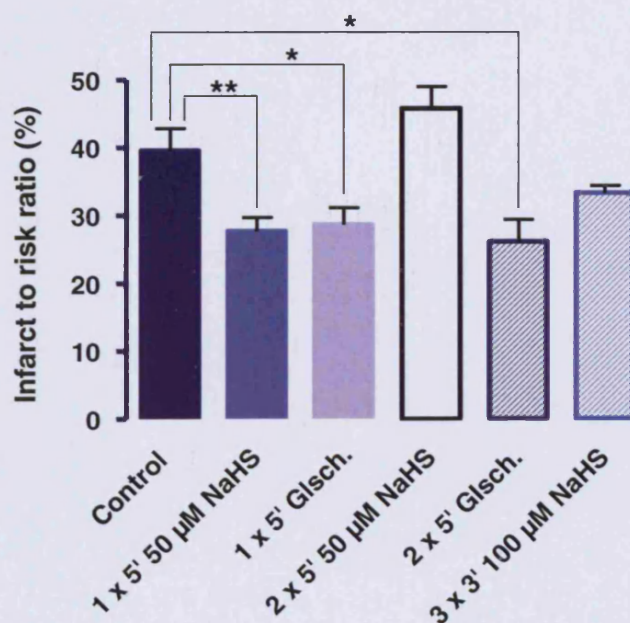


Figure 6.12 Comparison of infarct to risk ratios (%), between various NaHS and global ischaemia preconditioning protocols. A single NaHS preconditioning stimulus induced significant cardioprotection in comparison to multiple which caused no protective effect. Both global ischaemic protocols also significantly reduced infarct size. One-way ANOVA was used to test for significance differences between all groups, $*p < 0.05$, $**p < 0.01$. Each value shown is the mean \pm SEM from 4-15 individual hearts.

6.6.2.5 Coronary flow and contractility data for studies 3

Baseline cardiodynamic data (heart rate, coronary flow rate, developed pressure and rate pressure product for the hearts in study 3 are summarised in figure 6.13. The rate pressure product and coronary flow rate of the hearts throughout the experiments is presented in figure 6.14. There were no significant effects of any of the compounds tested on the measured parameters. Regional ischaemia caused a reduction in rate pressure product and coronary flow rate in all treatment groups as expected (as shown in figure 6.14). Run-down of the hearts is demonstrated by a reduction in rate pressure product in all treatment groups as expected (as shown in figure 6.14).

Treatment group	n	CFR ml/min	HR BPM	LVDP mmHg	RPP mmHg/min x10 ³	RV and LV vol. cm ³	Risk zone vol. cm ³
Control	15	17.7 ± 0.9	296 ± 16	112.3 ± 7.5	32.6 ± 2.1	0.84 ± 0.02	0.40 ± 0.03
1 x 5' 50 µM NaHS precon.	15	19.9 ± 1.1	277 ± 11	156.0 ± 21.6	30.7 ± 1.7	0.81 ± 0.03	0.40 ± 0.02
1 x 5' Glsch. precon.	14	18.0 ± 0.8	269 ± 13	106.0 ± 5.8	27.9 ± 1.4	0.79 ± 0.01	0.42 ± 0.02
2 x 5' 50 µM NaHS precon.	4	19.7 ± 0.6	308 ± 24	113.4 ± 14.6	33.7 ± 2.0	0.76 ± 0.02	0.42 ± 0.03
2 x 5' Glsch. precon.	6	17.8 ± 0.8	272 ± 19	131.7 ± 15.4	33.6 ± 3.6	0.84 ± 0.03	0.41 ± 0.02
3 x 5' 100 µM NaHS precon.	7	19.5 ± 1.0	259 ± 16	106.3 ± 8.2	27.8 ± 3.2	0.91 ± 0.03	0.47 ± 0.04

Figure 6.13 Baseline cardiodynamic data for hearts preconditioned with NaHS. Abbreviations: LV = left ventricle, RV = right ventricle, CFR = coronary flow rat, HR = heart rate, LVDP = left ventricular pressure, RPP = rate pressure product (HR x LVDP).

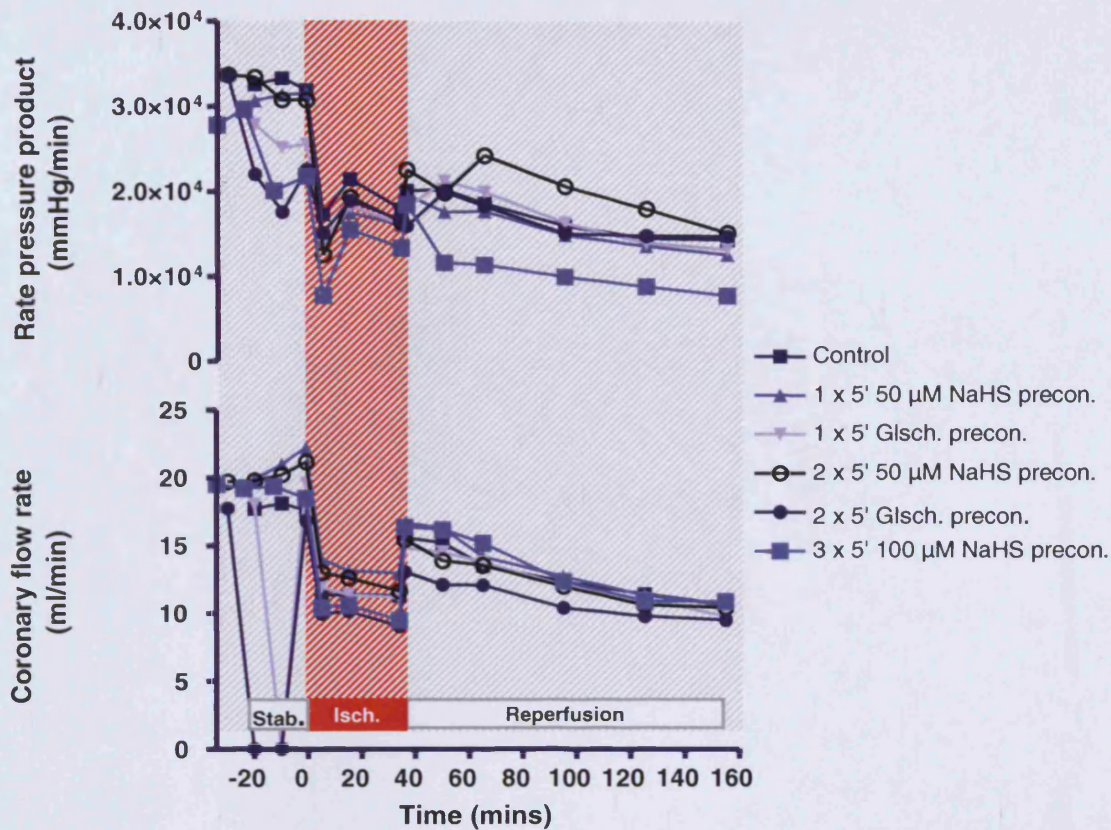


Figure 6.14 Rate pressure product (RPP) (A) and coronary flow rate (CFR) (B) for hearts preconditioned with NaHS. Stab.=stabilisation period (20 mins), Isch.=Regional ischaemia (35 mins), Reperfusion (120 mins). Each value shown is the mean from 6-15 individual hearts. SEM error bars have been removed for clarity.

6.6.2.6 Study 4: Effect of inhibition of PI3K on NaHS preconditioning

The protective effect of preconditioning with NaHS was attenuated in the presence of the PI3K inhibitor (LY294002) ($34.3 \pm 3.1\%$ vs. $27.7 \pm 2.1\%$, $**p<0.05$). LY294002 alone increased the infarct size above control ($47.9 \pm 3.6\%$ vs. $39.2 \pm 3.0\%$), although not significantly (data summarised in figure 6.15).

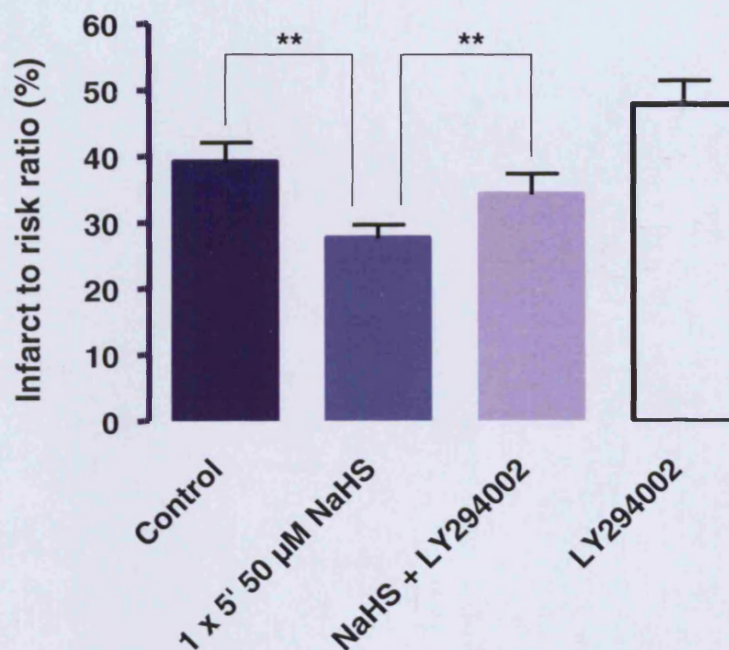


Figure 6.15 Comparison of infarct to risk ratio (%), for NaHS preconditioned hearts in the presence and absence of the PI3K inhibitor LY294002 10 µM.. LY294002 significantly attenuated the cardioprotective effect of NaHS preconditioning, and alone significantly increased infarct size. $**p<0.01$, one-way ANOVA was used to test for significance differences between all groups. Each value shown is the mean \pm SEM from 5-18 individual hearts.

6.6.2.7 Study 5: Effect of Akt inhibition on NaHS preconditioning

The protective effect of preconditioning with NaHS was attenuated in the presence of the Akt inhibitor (Akti VIII) ($36.6 \pm 5.3\%$ vs. $27.7 \pm 2.1\%$ for NaHS + Akti VIII and NaHS respectively). Akti VIII alone increased the damaged to the myocardium above the control ($45.3 \pm 5.7\%$ vs. $39.2 \pm 3.0\%$ for Akti VIII and Control respectively), although not significant (as shown in figure 6.16).

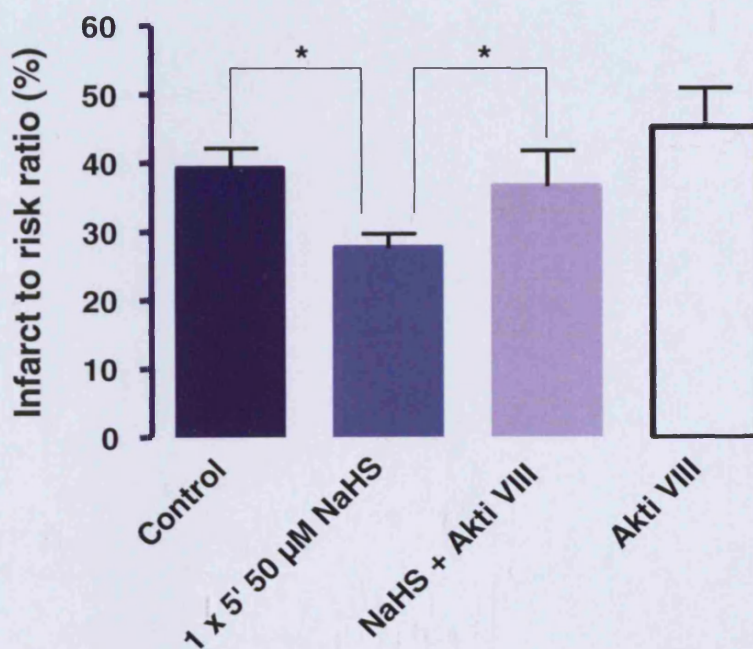


Figure 6.16 Comparison of infarct to risk ratio (%) for NaHS preconditioned hearts in the presence and absence of the Akt inhibitor Akti VIII 2 µM. Akti VIII significantly attenuated the cardioprotective effect of NaHS preconditioning, and alone significantly increased infarct size. * $p < 0.05$, one-way ANOVA was used to test for significance differences between all groups. Each value shown is the mean \pm SEM from 5-18 individual hearts.

6.6.2.8 Coronary flow and contractility data for studies 4 and 5

Baseline cardiodynamic data (heart rate, coronary flow rate, developed pressure and rate pressure product for the hearts in studies 4 and 5 are summarised in figure 6.17. The rate pressure product and coronary flow rate of the hearts throughout the two experiments is presented in figure 6.18. There were no significant effects of any of the compounds tested on the measured parameters. Regional ischaemia caused a reduction in rate pressure product and coronary flow rate in all treatment groups as expected (as shown in figure 6.18). Run-down of the hearts is demonstrated by a reduction in rate pressure product in all treatment groups as expected (as shown in figure 6.18).

Treatment group	n	CFR ml/min	HR BPM	LVDP mmHg	RPP mmHg/min x10 ³	RV and LV vol. cm ³	Risk zone vol. cm ³
Control (inc. DMSO n=3)	18	17.3 ± 0.8	297 ± 18	107.7 ± 7.0	31.8 ± 2.5	0.85 ± 0.02	0.39 ± 0.03
1 x 5' 50 µM NaHS precon.	15	19.9 ± 1.1	277 ± 11	156.0 ± 21.6	30.7 ± 1.7	0.81 ± 0.03	0.40 ± 0.02
NaHS + LY294002	5	18.6 ± 1.2	286 ± 9	124.0 ± 7.0	35.6 ± 2.6	0.93 ± 0.02	0.49 ± 0.03
LY294002	5	14.0 ± 1.9	283 ± 21	89.2 ± 11.3	24.7 ± 2.8	0.79 ± 0.01	0.42 ± 0.02
NaHS + Akti VIII	5	19.1 ± 2.8	288 ± 2.8	117.5 ± 9.5	35.2 ± 2.5	0.87 ± 0.04	0.48 ± 0.01
Akti VIII	5	21.1 ± 2.2	262 ± 23	93.4 ± 4.8	24.5 ± 2.7	0.89 ± 0.02	0.43 ± 0.02

Figure 6.17 Baseline cardiodynamic data for hearts preconditioned with NaHS in the presence and absence of a PI3K and Akt inhibitor. Abbreviations: LV = left ventricle, RV = right ventricle, CFR = coronary flow rat, HR = heart rate, LVDP = left ventricular pressure, RPP = rate pressure product (HR x LVDP).

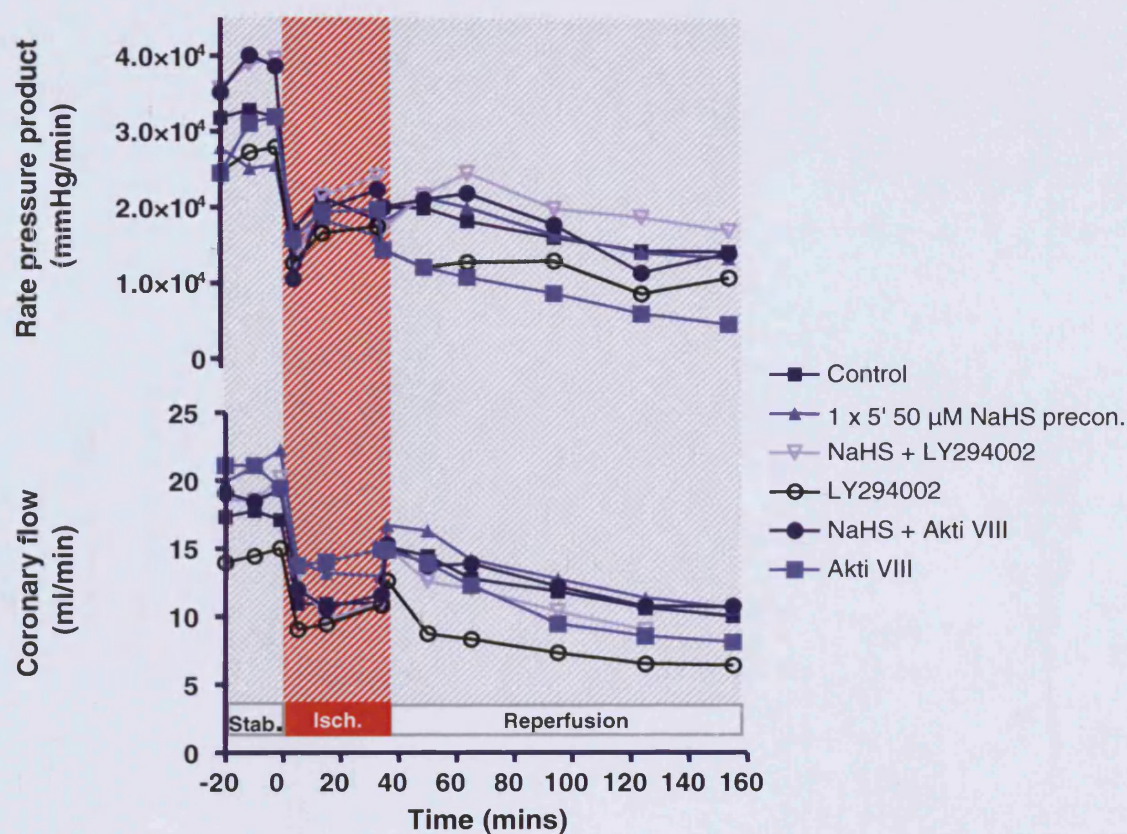


Figure 6.18 Rate pressure product (RPP) (A) and coronary flow rate (CFR) (B) for hearts preconditioned with NaHS in the presence and absence of a PI3K and Akt inhibitor. Stab.=stabilisation period (20 mins), Isch.=Regional ischaemia (35 mins), Reperfusion (120 mins). Each value shown is the mean from 5-18 individual hearts. SEM error bars have been removed for clarity.

6.6.3 MESNA data

6.6.3.1 Study 6: Pharmacological pre- and postconditioning of hearts with MESNA

Pharmacological preconditioning with MESNA 50 μ M produced cardioprotection with a significant reduction in infarct size compared to control ($27.1 \pm 2.1\%$ vs. $39.2 \pm 3.4\%$, $*p < 0.05$). A similar cardioprotective effect was seen with MESNA postconditioning compared to control ($26.1 \pm 3.5\%$ vs. $39.2 \pm 3.4\%$, $p < 0.05$) (data summarised in figure 6.19).

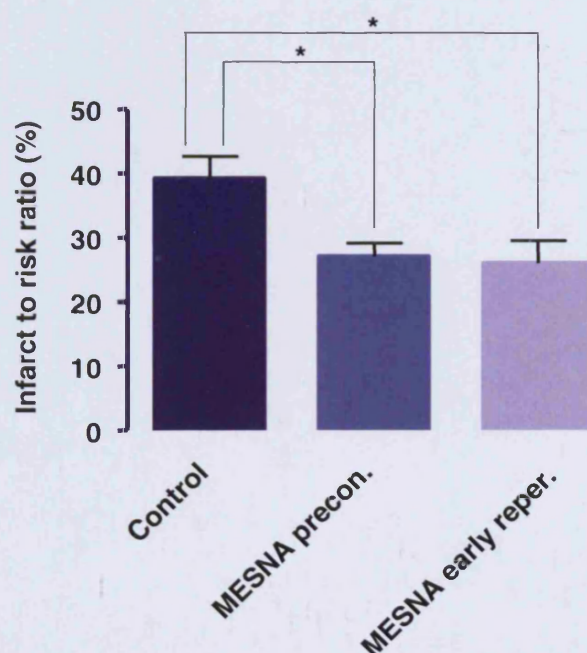


Figure 6.19 Comparison of infarct to risk ratio (%) for MESNA preconditioned and postconditioned hearts. Both pre- and postconditioned hearts produced a significant reduction in infarct size. $*p < 0.05$, one-way ANOVA was used to test for significance differences between all groups. Each value shown is the mean \pm SEM from 6-12 individual hearts.

6.6.3.2 Study 7: The effect of a PI3K inhibitor on the cardioprotective effect of MESNA at early reperfusion

The cardioprotective effect of MESNA at early reperfusion was attenuated in the presence of the PI3K inhibitor LY294002 10 μ M ($26.1 \pm 3.5\%$ vs. $40.6 \pm 2.5\%$, $*p < 0.05$). LY294002 alone also produced an increase in infarct size compared to control although this was not significant (48.6 ± 2.6 vs. $39.2 \pm 3.4\%$) (data summarised in figure 6.20).

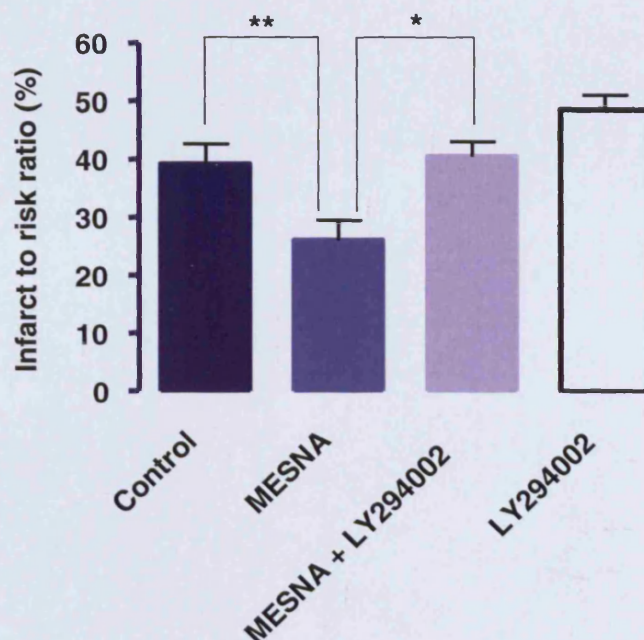


Figure 6.20 Comparison of infarct to risk ratio (%), for MESNA postconditioned hearts in the presence and absence of the PI3K inhibitor LY294002. LY294002 significantly attenuated the cardioprotective effect of MESNA postconditioning. * $p < 0.05$, one-way ANOVA was used to test for significance differences between all groups. Each value shown is the mean \pm SEM from 7-12 individual hearts.

6.6.3.3 Coronary flow and contractility data for studies 6 and 7

Baseline cardiodynamic data (heart rate, coronary flow rate, developed pressure and rate pressure product for the hearts in studies 6 and 7 are summarised in figure 6.21. The rate pressure product and coronary flow rate of the hearts throughout the two experiments is presented in figure 6.22. There were no significant effects of any of the compounds tested on the measured parameters. Regional ischaemia caused a reduction in rate pressure product and coronary flow rate in all treatment groups as expected (as shown in figure 6.22). Run-down of the hearts is demonstrated by a reduction in rate pressure product in all treatment groups as expected (as shown in figure 6.22).

Treatment group	n	CFR ml/min	HR BPM	LVDP mmHg	RPP mmHg/min x10 ³	RV and LV vol. cm ³	Risk zone vol. cm ³
Control	12	17.7 ± 0.9	276 ± 19	108.5 ± 9.4	30.1 ± 3.3	0.94 ± 0.04	0.46 ± 0.05
MESNA 50 µM precon.	6	18.7 ± 1.1	242 ± 8	116.4 ± 6.0	28.1 ± 1.6	0.94 ± 0.04	0.43 ± 0.03
MESNA 50 µM postcon.	6	20.6 ± 2.0	252 ± 22	134.4 ± 9.0	32.9 ± 1.2	0.90 ± 0.02	0.43 ± 0.02
MESNA postcon. + LY294002	7	19.0 ± 1.2	264 ± 15	166.2 ± 8.8	30.7 ± 2.6	1.05 ± 0.05	0.57 ± 0.06
LY294002 10 µM	7	16.0 ± 1.8	261 ± 20	111.8 ± 17.5	27.5 ± 2.9	0.83 ± 0.03	0.44 ± 0.02

Figure 6.21 Baseline cardiodynamic data for hearts used in the MESNA perfusion studies. Abbreviations: LV = left ventricle, RV = right ventricle, CFR = coronary flow rat, HR = heart rate, LVDP = left ventricular pressure, RPP = rate pressure product (HR x LVDP).

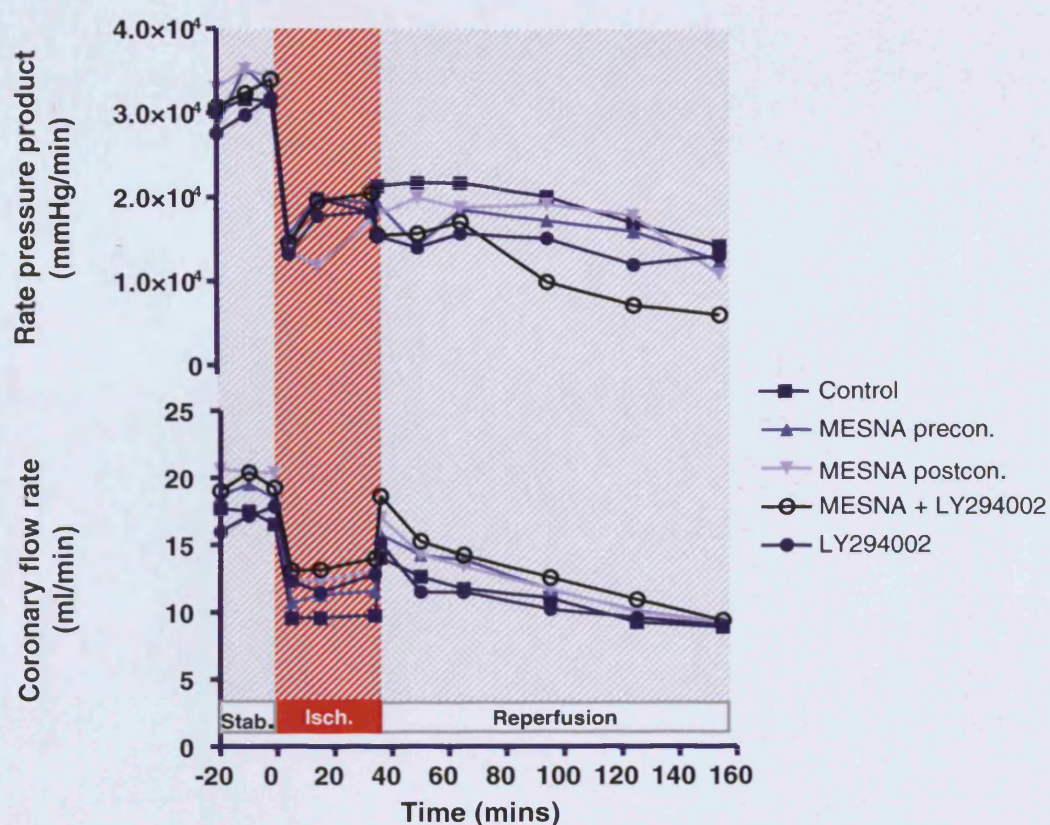


Figure 6.22 Rate pressure product (RPP) (A) and coronary flow rate (CFR) (B) for hearts used in the MESNA perfusion studies. Stab.=stabilisation period (20 mins), Isch.=Regional ischaemia (35 mins), Reperfusion (120 mins). Each value shown is the mean from 7-12 individual hearts. SEM error bars have been removed for clarity.

6.7 Discussion

The findings of these studies can be summarised briefly as follows:

1. Pharmacological preconditioning with NaHS was the only effective pharmacological protocol to induce cardioprotection in the Langendorff perfused isolated rat heart.
2. The infarct limiting effect of NaHS preconditioning was attenuated in the presence of the PI3K inhibitor LY294002 and the Akt inhibitor Akti VIII, suggesting a potential for NaHS to activate the RISK pathway to induce cardioprotection.
3. Pharmacological pre- and postconditioning with MESNA induced cardioprotection in isolated rat hearts.
4. The cardioprotective effect of MESNA postconditioning was abolished in the presence of LY294002, suggesting MESNA may activate the RISK pathway to induce cardioprotection.

6.7.1 Perfusion of hearts with NaHS

Perfusion of hearts with NaHS during early reperfusion does not induce cardioprotection

Initially in this study, hearts were perfused with NaHS either at early reperfusion, or during the early part of ischaemia. This approach was taken since it has previously been reported that isolated rat hearts perfused with NaHS (1 μ M) during ischaemia plus early reperfusion were found to be protected (Johansen *et al.*, 2006). However, it was not clear at what point H₂S was required to induce protection.

The results obtained in this study suggest that perfusing the heart with H₂S specifically at early reperfusion is not cardioprotective at the concentrations tested (1 – 50 μ M NaHS). It appears that the 10 μ M NaHS may show some tendency towards cardioprotection, but this is not a significant finding. Interestingly, the 50 μ M NaHS does not offer any further protection and matches the control infarct size. This highlights the fine homeostatic regulation of H₂S in the body, since it is reported that only a two-fold concentration above physiological concentrations is in fact toxic

(Warenycia *et al.*, 1989). Taken in this context, it is possible that there is a concentration of NaHS that lies between 10 and 50 μ M that offers protection.

There are several published studies that contradict the findings of the studies presented in this chapter and show a cardioprotective role of H₂S at reperfusion. Ji *et al.* (Ji *et al.*, 2008) showed that four, 15 second perfusions with NaHS (1 μ M) with 15 seconds of normal buffer in between at reperfusion, produced a marked concentration-dependent reduction in infarct size. Pharmacological postconditioning with NaHS improved cardiodynamics, and produced a reduction in infarct size in isolated rat hearts. These protective effects were abolished in the presence of a PI3K inhibitor or a PKC inhibitor (Yong *et al.*, 2008a), suggesting that H₂S stimulates PI3K and PKC signalling. Possible explanations for the differences observed between the studies presented in this chapter and published data, could be due to the severity of acidosis occurring during ischaemia-reperfusion effecting the dissociation of NaHS and subsequent availability of H₂S, and also variation in the susceptibility of some of the rat the hearts to myocardial damage effecting the amount of infarct size limitation achieved.

eNOS activity has been shown to be stimulated in hearts perfused continually with NaHS at reperfusion (Yong *et al.*, 2008a), suggesting that H₂S may up-regulate the production of NO, a central component of the RISK pathway in eliciting cardioprotection. Yong *et al.* (2008a) have shown that ischaemic postconditioning (6 episodes of 10 second ischaemia at the onset of reperfusion) stimulated the production of endogenous H₂S during the early period of reperfusion in isolated perfused rat hearts. In the presence of PAG, a selective CSE inhibitor, there was an attenuation of the improved contractile function and also a reduction in PKC and PKG phosphorylation.

In an *in vivo* mouse model Elrod *et al.* (2007) demonstrated the ability to induce cardioprotection with NaHS (50 μ g/kg) at reperfusion. They concluded that the cardioprotective effect of NaHS was through the preservation of mitochondrial functions. These findings suggest that after H₂S activates PI3K and Akt, it induces

cardioprotection via activation of the downstream components of the RISK pathway, preventing the opening of the mPTP. They also showed that transgenic mice with increased expression of CSE in the myocardium demonstrated the ability to produce twice as much H₂S as wild type mice. A 47% reduction in infarct size, compared to wild type control, has also been observed in *in vivo* hearts subjected to 45 minute left coronary artery occlusion and 72 hours of reperfusion (Elrod *et al.*, 2007). These findings suggest that upregulated endogenous H₂S production is cardioprotective and support the findings of Silvarajah *et al.* (2006) who demonstrated that inhibition of CSE caused increased myocardial damage in a rat model of ischaemia-reperfusion.

Perfusion of hearts with NaHS during early ischaemia did not induce cardioprotection

Hearts perfused with NaHS during the initial stages of ischaemia also showed no protection against ischaemia-reperfusion injury at the concentrations tested (1-10 µM NaHS). There was also a slight reduction in infarct size with 10 µM NaHS, as was seen with 10 µM NaHS at reperfusion, although this was not significant. A higher concentration of NaHS was not tested, since there was evidence that NaHS 50 µM was shown to offer no protection when hearts were perfused at reperfusion implying cytotoxic effects. Surprisingly, hearts perfused with NaHS 1 µM for the duration of ischaemia and early reperfusion showed no cardioprotection. There was not even a tendency towards a protective effect. These findings are in marked contrast to a pilot study performed in our lab prior to the start of my PhD, whereby perfusing isolated rat hearts for the duration of 35 minutes regional ischaemia and early reperfusion caused a significant reduction in infarct size compared to control (Johansen *et al.*, 2006). These findings do however support those of Bilksoen *et al.* (2008) who reported no significant reduction in infarct size in isolated rate hearts perfused with NaHS 40 µM for the duration of ischaemia and reperfusion. They accounted the lack of cardioprotection to two outlying infarct values, but experience of working with NaHS in our lab would suggest that this was simply the varying nature of the results obtained with NaHS. Additional contradictory data was generated in the study presented in this chapter with the H₂S donor NaHS. These are outlined in detail later in this discussion along with possible reasoning.

A single 5 minute NaHS preconditioning stimulus induced cardioprotection

A single 5 minute perfusion of NaHS with 5 minutes washout, prior to a 35 minute regional ischaemic episode, induced a significant reduction in the infarct size compared with control. This pharmacological preconditioning protocol also produced a similar reduction in infarct compared to its time matched control which received 5 minutes global ischaemia preconditioning. However, increasing the number of NaHS preconditioning stimuli to two episodes of 5 minutes, and three episodes of 3 minutes did not produce cardioprotection, unlike the time matched global ischaemia controls. As a result of these findings the single 5 minute NaHS preconditioning perfusion protocol was selected for the inhibitor studies. This cardioprotective effect was of a 5 minute NaHS preconditioning perfusion was abolished in the presence of either the PI3K inhibitor, LY294002, or an Akt inhibitor, Akti VIII, at reperfusion. These findings suggest that H₂S may act via these initial upstream components of the RISK pathway to induce cardioprotection at reperfusion.

Interestingly a paper published by Hu *et al.* (2008) showed that preconditioning with NaHS (100 μ M) for 3, 3 minute cycles prior to a prolonged ischaemic episode induced significant myocardial protection. This was contradictory with the findings from the data presented in this chapter which showed no cardioprotection using this protocol. However, in keeping with the findings presented in this chapter of the involvement of PI3K and Akt in NaHS' cardioprotection, the authors demonstrated that the protective effect of NaHS (three time 3 minute episodes NaHS 100 μ M) was abolished in the presence of LY294002 (15 μ M) during both preconditioning and ischaemia. Preconditioning with NaHS has also been shown to increase the phosphorylation levels of Akt, suggesting that NaHS triggered cardiac protection via the PI3K/Akt upstream components of the RISK pathway (Hu *et al.*, 2008).

PKC has also been implicated in the cytoprotective signalling cascade activated by H₂S. Preconditioning with NaHS activated PKC α , ϵ , and δ isoforms (Pan *et al.*, 2008). In the presence of glibenclamide translocation of the ϵ and δ isoforms to the membrane fraction was abolished, suggesting an involvement of the K_{ATP} channel in their

activation. NaHS preconditioning also significantly accelerated the decay of electrically and caffeine induced intracellular calcium transients, which was reversed in the presence of the selective PKC inhibitor chelerythrine. These findings suggest that preconditioning with NaHS activates PKC in cardiomyocytes resulting in accelerated clearance of intracellular calcium by stimulating the sodium/calcium exchanger and sarcoplasmic reticulum calcium ATP-ase mechanisms (Pan *et al.*, 2008).

Oxidative stress may explain the lack of cardioprotection seen with NaHS at early ischaemia and early reperfusion

The lack of protection seen with NaHS at early reperfusion and early ischaemia in the isolated rat heart, may be explained by large amount of oxidative stress already occurring in the heart as a result of free radical accumulation during this period (Goldhaber *et al.*, 1992). Oxidative stress occurs as a result of an imbalance between oxidant and antioxidant defence systems, resulting in cell damage (Cesselli *et al.*, 2001). Since it is known that H₂S has a toxic profile, the free radical characteristic of the compound may be exacerbated during periods of ischaemia-reperfusion, as opposed to providing a protective action. H₂S appears to have a very narrow therapeutic index for protection. Endogenous levels are tightly regulated, since there is only a 2-fold difference between a physiological and a toxic level (Warenycia *et al.*, 1989). H₂S may therefore be protective when given as a preconditioning stimulus as a result controlling the exposure level of the gas to cells, since a single 5 minute exposure followed by washout is all that the cells were exposed to. As a result there is the reduced probability that the gas will accumulate and cause detrimental effects. In addition, during the basal perfusion period the heart is not undergoing high levels of oxidative stress and H₂S may therefore be able to act to pharmacologically precondition since cells are in a more receptive state than they are during ischaemia-reperfusion. ATP is also present to drive active processes enabling the RISK pathway to be primed. Increasing the number of NaHS preconditioning stimuli produced no further increase in cardioprotection. In fact two, 5 minute NaHS preconditioning stimuli increased the infarct size above control, although not statistically significant. This again highlights the fine balance between the cardioprotective and deleterious effect of H₂S.

Another possible explanation for the lack of protective efficacy seen with H₂S during ischaemia and reperfusion, particularly with the lower concentrations tested (1 and 10 μ M), is that such a low concentration of derived H₂S was present. Indeed, Johansen *et al.* (2006) showed that over a 55 minute perfusion with NaHS (1 μ M) during the duration of ischaemia and start of reperfusion, cardiac protection was induced. Since H₂S is such a volatile compound that is readily broken down, the 15 minute perfusions performed within this study were perhaps simply not long enough at the concentrations tested to induce protection. H₂S may accumulate within the heart and only when a certain threshold concentration is reached does protection occur. Increasing the concentration of NaHS to 50 μ M showed no protective effect. Johansen *et al.* (2006) also showed that increasing the concentration of NaHS from 1 μ M to 10 μ M over ischaemia and early reperfusion, showed no protection and was associated with cytotoxic, metabolic or other non-specific effects. This illustrates the very balance between the gas' protective and deleterious effect.

It has also been shown that direct administration of NaHS (50 μ g/kg) to the heart at reperfusion in an *in vivo* mouse model also caused substantial protection to the myocardium shown by a reduction in infarct size (Elrod *et al.*, 2007). However, since this was an *in vivo* model, there may have been other endogenous factors present that influenced the tissue availability of the free gas, making it difficult to compare the findings to the *ex vivo* model used in this study.

6.7.1.1 Difficulties of working with the H₂S donor NaHS

Other groups investigating the actions of H₂S have commented on the difficulty of working with such an unstable compound (Li *et al.*, 2008a; Zhao *et al.*, 2002) and the need for additional chemical tools to assist in understanding the complex biological roles of H₂S in the body. The major difficulties of working with NaHS as an H₂S donor lie within determination of the final concentration of H₂S, the storage of the compound both as a solid and in solution, and also the apparent lack of awareness of some of the users when publishing in the literature as to whether the concentration that they refer to is of H₂S or the parent compound NaHS.

The pH of the internal environment of the heart may have effected the dissociation of the NaHS to form H₂S. When NaHS dissociates in water it forms Na⁺ and HS⁻. The HS⁻ then associates with H⁺ to form H₂S. In solution at pH 7.4 and 37 °C, approximately 18.5% of H₂S exists in the undissociated form H₂S and the remainder exists as HS⁻ which is at equilibrium with H₂S (Dombkowski *et al.*, 2004). During ischaemia it is known that intraceullular acidosis occurs as a result of accumulation of metabolic waste products (Kaski *et al.*, 1998). This may effect the final concentration of H₂S which is present by altering the dissociation constant of NaHS predicted by the Henderson Hasselbach equation. The extracellular pH may not be to different as a result of the buffering action of the Krebs-Henseleit buffer.

The stability of H₂S in solution has also been investigated (Zhao *et al.*, 2002). It appears that the stability of H₂S in solution depends on the starting concentration of H₂S. A 1mM solution of pure bubbled H₂S gas has been shown to drop by 15% in 30 minutes. The graph in figure 6.23 demonstrates that at lower starting concentrations of H₂S, there is less decline in the concentration remaining in solution. This provides confidence that suggesting that in the concentrations tested the H₂S should have been relatively stable during the short perfusion periods of up to 15 minute. This is assuming that H₂S derived from NaHS acts in the same manner in solution as the pure bubbled gas.

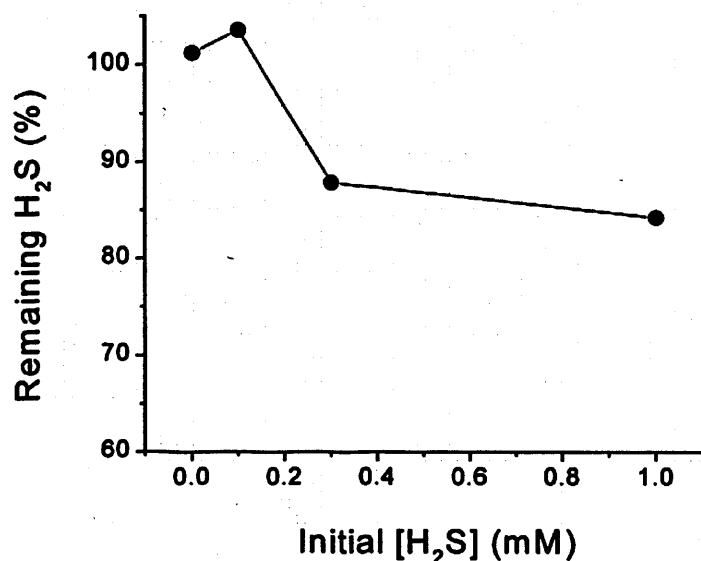


Figure 6.23 Stability of H₂S solution in Krebs-Henseleit buffer solution. H₂S concentrations in the Krebs-Henseleit buffer were measured immediately after they were made and 30 minutes afterwards. The amount of remaining H₂S is expressed as a percentage of the initial measured concentration. Graph from Zhao *et al.* (2002).

6.7.1.2 Alternative H₂S donor compounds

Another potentially viable option to NaHS is to bubble pure H₂S gas into solution. Whilst this ensures that only H₂S is present in solution, it does not enable the final concentration of the solution to be easily measured. There are H₂S probes widely used for environmental detection of H₂S, but these do not appear to be sensitive enough to detect the concentrations within the desired detection range. Other compounds have been synthesised such as S-diclofenac, which is a non-steroidal anti-inflammatory drug (NSAID) with a sulphur group attached. It has been shown that it is possible to induce marked cardioprotection in global low-flow ischaemia in the isolated rabbit heart using the H₂S donor compound, S-diclofenac, when compared it the parent compound diclofenac (Rossoni *et al.*, 2008). As yet there appear to be no studies conducted in the isolated rat heart using regional ischaemia, but it would be interesting to see the effectiveness of this H₂S donor and whether it is a viable alternative to NaHS. Another H₂S donor compound called Lawesson's compound has also recently emerged into the literature (Li *et al.*, 2008a). Lawesson's compound is a thionation reagent which as yet does not appear to have been used in ischaemia-

reperfusion experiments, but may have promising potential as an alternative to NaHS in the future.

6.7.1.3 Further studies with NaHS

It would be interesting to investigate the effects of blocking further downstream components of the RISK pathway, in particular investigating the potential interaction of H₂S with NO synthesis component of the RISK pathway. It has been reported that there is an interaction between NO and H₂S, since the production of both gases can be increased by pro-inflammatory mediators of haemorrhagic shock (Mok *et al.*, 2004) and bacterial lipopolysaccharide (Li *et al.*, 2005). H₂S has been shown to both enhance and attenuate the relaxant effect of NO in the rat aorta, whilst NO has been shown to enhance the release of H₂S. These contradictory findings may it difficult to define the interaction of the gases. More recently it has been shown that NO and peroxynitrite react with H₂S to form a novel nitrosothiol, which has been proposed to regulate the physiological effects of NO and H₂S (Whiteman *et al.*, 2006). Experiments giving an NO synthase inhibitor, such as L-NAME at reperfusion, in H₂S preconditioned Langendorff perfused isolated rat hearts, would be useful to assist in the understanding of the interaction of the two gases. Western blotting studies to compare the phosphorylation state of eNOS in H₂S preconditioned and control hearts would also be beneficial.

In aortic tissue H₂S may directly interact with the K_{ATP} channels to cause hyperpolarisation and relaxation (Zhao *et al.*, 2001a). This suggests that H₂S has the potential to act directly on the K_{ATP} component of the RISK pathway and may mediate protection by activating components downstream of the ion channel. Alternatively after activation of PI3K and Akt by H₂S, alternative pathways may be activated that induce cardioprotection. For example Pim-1, a proto-oncogenic serine-threonine kinase (Wang *et al.*, 2001), has been shown to regulate cardiomyocyte survival downstream of Akt by regulating cell survival and proliferation (Muraski *et al.*, 2007). The role of Pim-1 in cardioprotection is still an emerging field. It is also known that the PI3K inhibitor LY29002, binds to and inhibits Pim-1 (Jacobs *et al.*, 2005), suggesting

that previous studies with LY294002 may require reinterpretation in the context of Akt dependent Pim-1 signalling (Muraski *et al.*, 2007).

Ideally a protective therapeutic intervention after a cardiovascular event would occur at reperfusion, since it is not possible to predict when a heart attack may occur. In this series of experiments H₂S did not induce cardioprotection at reperfusion, but the ability to pharmacologically precondition the heart suggests that there is still a potential therapeutic role. For example in situations such as episodes of unstable angina or prior to cardiac interventions such as angioplasty, when it is known that the heart is going to be at risk of a scheduled ischaemic period, administration of H₂S to prime the RISK pathway and reduce the subsequent damage to the heart may be possible. H₂S as a cardioprotective agent is still an emerging field and with the appearance of improved pharmacological tools, in particular H₂S donors, knowledge of the field should improve more rapidly.

6.7.2 Perfusion of hearts with MESNA

MESNA induces cardioprotection as a preconditioning stimulus and at early reperfusion

The results from MESNA perfused hearts show that cardioprotection can be induced when hearts were either pharmacologically preconditioned with MESNA, or perfused with MESNA at early reperfusion with MESNA, with both protocols producing a significant reduction in infarct size compared to control.

MESNA at early reperfusion involves activation of PI3K

It was decided that the postconditioning effect of MESNA would be investigated in relation to the RISK pathway as opposed to pharmacological preconditioning with MESNA. This was because it was thought that reperfusion was the most clinically relevant therapeutic intervention period, due to unpredictable nature of the onset of myocardial infarction. In the presence of the selective PI3K inhibitor LY294002, the cardioprotective effect of MESNA was attenuated. This finding suggests that, at least

in part, MESNA may act to induce cardioprotection via activation of PI3K and subsequently activate the downstream components of the RISK pathway.

MESNA offers a more robust cardioprotection than NaHS

What was perhaps the most interesting observation when working with MESNA was the robustness of the cardioprotection elicited compared to NaHS. The cardioprotective effects of MESNA were investigated due to the difficulties of obtaining robust cardioprotection with NaHS and the distinct absence of alternative H₂S donors. MESNA is not strictly an H₂S donating compound, but it is a thiol (-SH) containing compound, meaning that it has the potential to act as a free radical scavenger and thus induce cardioprotection from ischaemia-reperfusion injury (Gressier *et al.*, 1994). On first observation of the mean infarct size values obtained with NaHS ($27.7 \pm 2.1\%$) and MESNA preconditioning ($27.1 \pm 2.1\%$) it would appear that both compounds produce the same level of cardioprotection. However, when the sample size of each of the groups is taken into account NaHS n=15 vs. MESNA n=6, it suggests that preconditioning the hearts with MESNA produced more robust cardioprotection since the SEM from a sample size of 6 is the same as that obtained from a sample size of 15. This implies that there was less spread of data around the mean and therefore a more consistent level of cardioprotection was obtained.

The cardioprotective actions of MESNA appear to be novel findings

The findings of this study appear to be first report of cardioprotection with MESNA in the ischaemic rat heart. This may be attributed to the fact the MESNA is primarily used in chemotherapy regimens for the prevention of hemorrhagic cystitis induced by the oxazaphosphorines cyclophosphamide and ifosfamide (Dechant *et al.*, 1991). Aside from MESNA's uroprotective ability, it has also been shown to protect the intestinal mucosa by reducing the toxicity of cisplatin (Allan *et al.*, 1986) and ifosfamide (Ypsilantis *et al.*, 2004) or reducing intestinal inflammation in experimental colitis (Shusterman *et al.*, 2003). Other published studies appear to be involved with investigating the protective effects of MESNA in ischaemia-reperfusion in the liver, kidney, and intestine (Ypsilantis *et al.*, 2006). MESNA's thiol group is thought to be

responsible for its antioxidant property which enables it to act as a free radical scavenger and thus limiting the damage associated with ischaemia-reperfusion when antioxidant levels are significantly increased (Gressier *et al.*, 1994). MESNA is also a small molecule which enables it to concentrate in the kidneys and therefore increase its ability as an anti-oxidant free radical scavenger (Gressier *et al.*, 1994). The ability of MESNA to accumulate may also exist in the heart, resulting in increased effectiveness of the compound and also lower concentrations required to produce effective cardioprotection whilst minimising the side effects or interacts with other drugs.

Not all thiols induce cardioprotection

Interestingly a study performed using the potential antioxidant drugs taurine, which contains a thiol group, was only shown to be of moderate effect at increasing external heart work after neutrophil-dependent reperfusion injury in an isolated guinea pig working heart model subjected to 15 minutes global ischaemia (Raschke *et al.*, 1995). A possible explanation for the lack of efficacy of this compound may be due to its size or charge, resulting in an inability to access the intracellular environment where its protective action was required. The potential explanations for the modest ability of taurine to induce cardioprotection may also apply to NaHS. Indeed NaHS may not have been acting as an H₂S donor, but in fact as an antioxidant to scavenge free radicals generated by ischaemia-reperfusion. Despite being the simplest structural form of a thiol and therefore the smallest molecule size, it is often described as a highly reactive thiol. Therefore it may have never reacted at the optimum intracellular site of action as a result of reacting with other species before it reached there, or never reaching there because it became too large a molecule as a result of reacting with other species.

6.7.2.1 Further studies with MESNA

Had time permitted it would have been interesting to investigate the effect of inhibiting other downstream components of the RISK pathway such as Akt, soluble guanylate cyclase, and PKG after hearts were either pre- or postconditioned with MESNA. This would have given a greater insight into whether MESNA acts via

activation of the RISK pathway or is simply a free radical scavenger to induce cardioprotection.

Another series of experiments that could be undertaken to further decipher the mechanism of action of MESNA would be to use an analogue compound with the thiol (-SH) group replaced by an unreactive alcohol group (-OH), such as Sodium 2-hydroxyethanesulphanate. Langendorff isolated perfused rat hearts could be perfused with the alternative compound and the effect on infarct size observed. If indeed MESNA's cardioprotective action is dependent on the thiol group, perhaps to act as a free radical scavenger, then removal of the group should result in the loss of cardioprotection.

6.8 Conclusion

The findings of these studies suggest that preconditioning the myocardium with the simple thiol containing compound NaHS induces protection against a subsequent ischaemia-reperfusion episode, with a mechanism that appears to involve activation of the P13Kinase and Akt components of the RISK pathway at reperfusion. The more complex thiol containing compound MESNA has also been shown to induce cardioprotection in a manner similar to that of NaHS, but with the addition of being also been cardioprotective when given at early reperfusion. MESNA produced a far more robust and consistent cardioprotection than NaHS. These two attributes of the MESNA compound are far more desirable and would enable it to be a more effective cardioprotective strategy given the volatile and unpredictable nature of the occurrence of myocardial infarction. What still remains unanswered though is exactly how these two thiols are producing the cardioprotection. An analogue of MESNA with the thiol group replaced with an inert moiety such as an alcohol group, would provide a far better insight into whether it is the derivation of H₂S gas that is producing the cardioprotection or the highly reactive thiol group acting as a free radical scavenger to limit infarct. The current findings do at least demonstrate further strong evidence to support H₂S as a cardioprotective mediator, even without a true understanding of the exact cardioprotective role of these compounds.

Chapter 7 General discussion

7.1 Principal findings:

The principal findings of the work presented in this thesis can be briefly summarised as follows:

1. Rat myocardium has the potential to express CSE and CBS protein, due to the confirmed detection of mRNA for both of these enzymes. It has not been possible to conclude with confidence if CSE and CBS protein is expressed in the rat myocardium, but it appears highly likely.
2. It is possible to exogenously stimulate the CSE enzyme, with its substrate L-cysteine, to produce H₂S gas which limits infarct size during regional ischaemia-reperfusion.
3. Basal H₂S levels have been shown to be up-regulated during ischaemia-reperfusion, consistent with an endogenous protective role within the myocardium
4. Simple and complex thiol containing compounds have been shown to be cardioprotective during regional ischaemia-reperfusion, with a mechanism that involves PI3K and Akt activation, implicating recruitment of downstream kinases within the RISK pathway.

7.2 Context and outlook

The outcomes of the all the studies conducted provide further characterisation of the roles and mechanisms of action of H₂S in the myocardium. They build upon previous findings, providing further evidence to support the role of H₂S as a highly effective endogenous and exogenous cardioprotective agent. Perhaps one of the most significant technical issues is the difficulty of working with NaHS, as an H₂S donor, due to the inability to consistently replicate both within my own data and previously published work. From a clinical aspect the finding that the administration of NaHS at reperfusion did not show cardioprotection is disappointing due to reperfusion being the first opportunity for medical intervention after AMI. However, other published studies have shown that NaHS is cardioprotective at reperfusion,

suggesting that the lack of cardioprotection may be due to the difficulties of working with NaHS. Interestingly other researchers are experiencing similar problems with NaHS and seeking alternative more robust compounds (Li *et al.*, 2008b; Zhao *et al.*, 2002). There is also increasing interest in trying to discover a compound that behaves more physiologically like H₂S, with a low dissociation rate (Li *et al.*, 2008b). The use of such tools would certainly improve not only the consistency of results, but also provide a more physiologically relevant compound to work with. The finding that MESNA was cardioprotective at reperfusion is of greater clinical relevance for the treatment of AMI, but great ambiguity surrounds which structural component of H₂S donors/thiol containing compounds produces cardioprotection. Further pharmacological characterisation is clearly needed and would greatly aid future mechanistic studies.

The limited success of the Western blotting studies, attributed to the poor selectivity of the antibodies, also highlights a need for better experimental tools to advance progress. More specific antibodies would also enable techniques such as immunohistochemistry to be performed, which would provide greater information about the distribution of the enzymes within organs and may assist with the discovery of a pharmacological target. However, a large amount of experimental evidence was obtained to support the existence of the H₂S synthesising enzymes within the myocardium from the pharmacological and biochemical studies. The data presented in chapter 4 demonstrate that exogenous stimulation of H₂S production resulted in cardioprotection, at least in part in a CSE dependent manner since the effect was attenuated by PAG. Furthermore, the data presented in chapter 5 demonstrated that tissue H₂S levels could be assayed and quantified, and were shown to be up-regulated during ischaemia-reperfusion. In addition measurement of the maximal H₂S synthesising capacity by exogenous stimulation showed that it was possible to generate H₂S within the myocardium. Ischaemia-reperfusion was also shown to reduce the ability of the left ventricle to generate H₂S, but evidence suggests that the right ventricle may increase production in a compensatory attempt to limit infarct size. These studies also highlighted that H₂S may also be generated

from an alternative enzyme to CSE, such as CBS or even a non-enzymatic source, since H_2S levels have been shown to increase after pharmacological inhibition of the CSE enzyme. Taken together these findings imply that H_2S acts as an endogenous cardioprotective agent and a feasible target for manipulation to protect the heart during ischaemia-reperfusion.

7.3 Proposed cardioprotection mechanism of action of thiol containing compounds

The ambiguity surrounding the cardioprotective mechanism of action of the thiol containing compounds, NaHS and MESNA, gives rise to two possible mechanistic explanations (summarised in figure 7.1). The first is based on the observation from this study that both compounds appear to signal through PI3K and Akt components of the RISK pathway, since pharmacological inhibition attenuated cardioprotection. It then follows that downstream components of the RISK pathway such as eNOS, soluble guanylate cyclase, and PKG are activated, ultimately resulting in inhibition of the mPTP and cardioprotection. This cardioprotective mechanism of action could be tested using Western blotting studies to measure the levels of phosphorylation of key components of the RISK pathway in the presence and absence of thiol stimulation. In particular investigating the phosphorylation of eNOS may provide further understanding of the interaction of H_2S and NO.

A perhaps less obvious, but equally valid proposed mechanism of action of NaHS and MESNA is that due to the highly reactive nature of thiols, they act as extremely effective free radical scavengers. This could occur in two ways. The first is to act independently of the RISK pathway, and instead reacting directly with free radicals and preventing them from causing damage to the myocardium through oxidative stress. Alternatively, and perhaps more in keeping with the observed findings, the thiol containing compounds may act as free radical scavengers and prevent ROS activation of Rho-kinase. Inhibition of Rho-kinase results in un-moderated activation of the RISK pathway, because Rho-kinase acts as an endogenous regulatory mechanism of the RISK pathway, possibly by phosphorylating phosphatase and

tensin homolog (PTEN). PI3K and Akt are therefore still required to enable the downstream components of the RISK pathway to be activated, hence why their inhibition attenuated cardioprotection. However, NaHS and MESNA act indirectly to activate the RISK pathway by inhibiting Rho-kinase and thus enabling the RISK pathway can become fully activated to induce cardioprotection. This proposed mechanism could be investigated using Western blotting techniques to measure the phosphorylation of Rho-kinase in the presence and absence of thiol containing compounds to determine if free radical scavenging prevents ROS induced activation of Rho-kinase. If in indeed this was the mechanism of action by which the thiols act, it may explain why MESNA provides more robust cardioprotection than NaHS. Perhaps the highly reactive nature of NaHS readily results in the formation of H₂S and thus renders it less effective as a ROS scavenging thiol. Indeed, MESNA is known for its stability and ability to accumulate in tissues, suggesting it may act as a more robust thiol containing compound and is therefore more effective as a free radical scavenger.

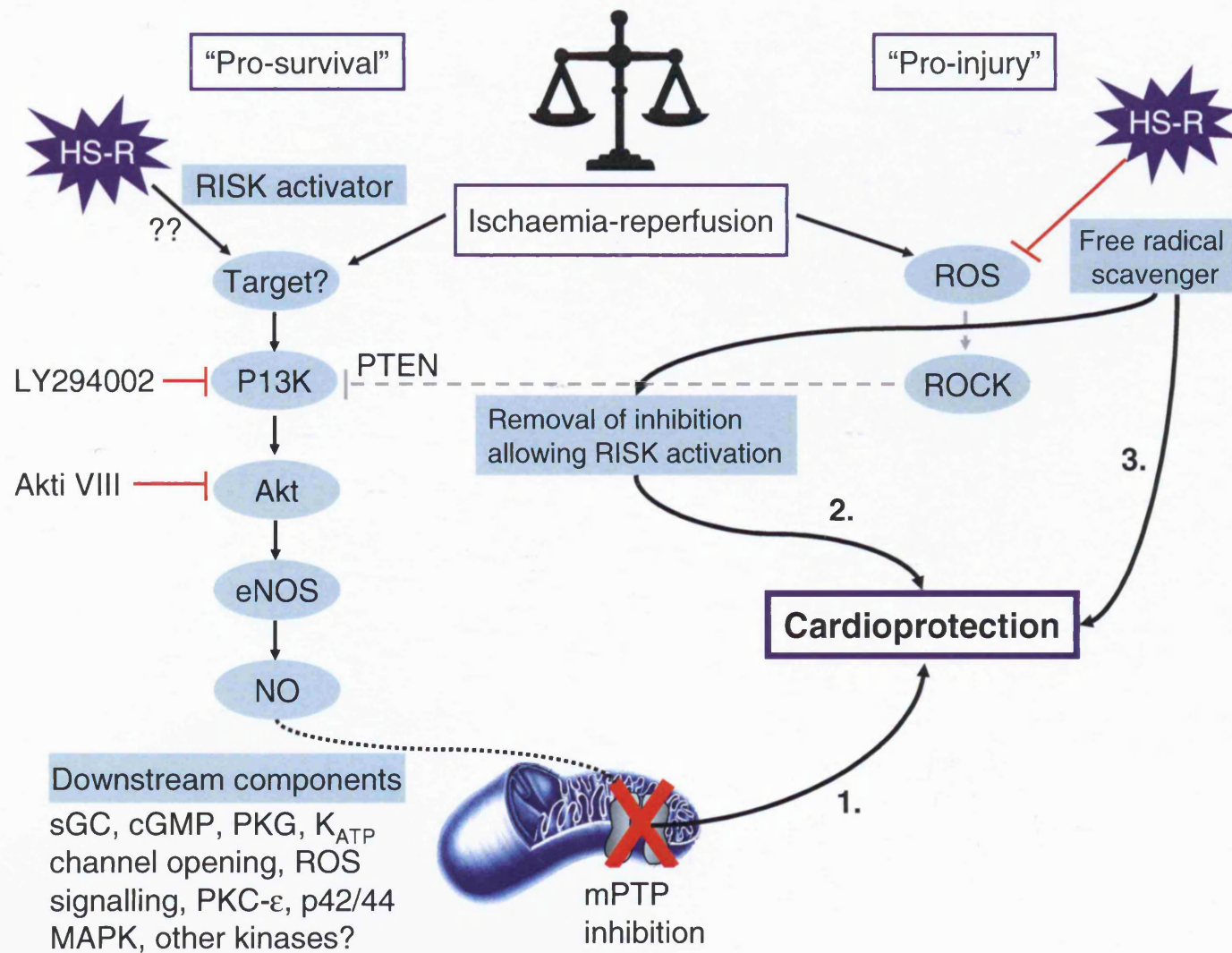


Figure 7.1 Summary of the three potential mechanism by which thiol containing/H₂S donor compounds may induce cardioprotection. 1. Direct activation of the RISK pathway occurs via an unknown target. 2. Free radical scavenging prevents activation of Rho-kinase via ROS, allowing unregulated activation of the RISK pathway. 3. Free radical scavenging produces cardioprotection by preventing oxidative stress damages independent of the RISK pathway.

7.4 Limitations of the thesis and scope for further studies

As with any scientific study, time is one of the main limiting factors and there is scope for further work. All of the studies conducted here are complimentary to each other and show a logical order of progression with each building upon previous findings. During the discussion to each chapter suggestions for further experiments have been proposed to complement the data already obtained. However, going beyond the obvious additional experiments and broadening the scope of this thesis are several other studies that would be of great interest and importance for characterising the cardioprotective role of H₂S in the myocardium.

Electrophysiology could be used to investigate the effect of H₂S on ion channels and electrical currents generated within isolated cardiac myocytes. There appears to be a distinct lack of knowledge of the mechanism of action of H₂S both as a cardioprotective agent and a vasodilator. Understanding more about how it influences ion channels and electrical currents within cells may assist in identifying other therapeutic interventions to influence its effect and may even provide information about a possible pharmacological target of H₂S. There is limited evidence in the literature that H₂S mediates its effect through opening of the K_{ATP} channel. This could be investigated further by patch clamping isolated cardiac myocytes and measuring the change in voltage when an H₂S donor was placed into the cell bathing media. A specific K_{ATP} channel blocker could be used and the effect on membrane potential observed. Another experiment could be conducted that exogenously stimulates the production of H₂S using the synthesising enzymes substrate L-cysteine and the effect on membrane potential measured. The synthesis of H₂S could be confirmed by the use of specific inhibitors to the enzymes. These experiments could be undertaken with relative ease and would provide much needed information about the effects of H₂S in the cell and how it mediates its cardioprotective effects.

Further studies investigating the downstream effectors of the RISK pathway would provide extremely important information about the mechanism of action of H₂S in cardioprotection. Whilst the results of the Langendorff isolated rat heart perfusion

studies have shown that PI3K and Akt appear to be involved in cardioprotection, these kinases are a common feature of many other signalling pathways and therefore it cannot be assumed that the remainder of the RISK pathway components are activated. The use of specific inhibitors to eNOS, soluble guanylate cyclase, PKG and the K_{ATP} channel given at the time of reperfusion either after preconditioning the hearts with an H_2S donor or giving an H_2S donor at reperfusion with the inhibitors could be investigated to decipher their involvement.

Identifying exactly how H_2S is generated in the myocardium is also of great importance. In the literature it is heavily reported that CSE is the key enzyme to produce H_2S in the myocardium, but as mentioned previously these findings lack convincing experimental evidence. There now arises the question that perhaps alternative enzymatic and non-enzymatic sources of H_2S may exist in the myocardium that act in a compensatory manner to protect the heart if the primary source is impaired. Perhaps the employment of a non-enzymatic source occurs during ischaemia when ATP levels are limited. Understanding how these H_2S sources interact and contribute to maintaining or elevating H_2S levels would provide a greater understanding of the protective mechanism of H_2S and the regulation of its production.

The limitations of the experimental techniques adopted have been highlighted and discussed at the end of each experimental chapter. Overall, the major limitation of the work presented in this thesis is the use of the rat as a species of choice to model potential therapeutic intervention in humans. However, rats are relatively cheap to buy, are abundantly available, and the Langendorff perfusion technique provides a robust model to investigate ischaemia-reperfusion. Also, the homology of CSE and CBS protein between rat and human has been shown to be very strong at 85.6 % (Ishii *et al.*, 2004) and 90 % (Kraus *et al.*, 1993) respectively, which enables the results from the enzyme characterisation in the rat to be confidently translated into the human. Moreover, the majority of published studies use the rat heart to model ischaemia-reperfusion, enabling results to be compared to those previously obtained. The dog is

also known to be a suitable model of ischaemia-reperfusion, having a similar physiology to the human. However, working with dogs would be far more expensive than rats, since everything is on a far larger scale. This would mean that greater amounts of drugs and reagents would be required, as well as more expensive purchasing and housing costs.

The main limitation with any pharmacological study is the specificity of the compounds used. Careful consideration to specificity was given when selecting the inhibitors and the experimental concentration used was based on approximately ten times the IC_{50} concentration, to ensure that they produced specific inhibition of the desired target. Where possible the most potent, selective and stable compounds were selected for a target. For example wortmanin is traditionally used for the inhibition of PI3K but it is known to be of limited selectivity and poor stability. Instead LY294002 was selected because it is more potent, specific, and stable. Ideally, at least two different compounds should have been used for each target, but this has serious cost and time implications. A more modern approach to pharmacological studies is to use silenced RNA (SiRNA) gene knock down mice models to ensure that the target of interest is specifically inhibited. Alternatively transgenic overexpression of a gene of interest can also be used in functional genomic studies. Both of these techniques have been shown to be very successful and the increased emergence of the use of the mouse heart for Langendorff studies has received increasing interest. However, knock-out breeding can be a slow process and the mouse is less physiologically relevant to the human than the rat.

7.5 Concluding remarks

The research conducted and presented in this thesis aimed to further the understanding of the roles and mechanisms of action of H_2S in the myocardium and its involvement in ischaemia-reperfusion. On balance I feel that the results obtained have provided a significant advancement in understanding the involvement of H_2S in cardioprotection during ischaemia-reperfusion. It has also raised many questions such as the exact mechanism of action of H_2S donor/thiol containing compounds and

highlighted the need for more robust H₂S donors, which would be a major contributing factor to accelerate the understanding of H₂S in ischaemia-reperfusion further.

The scope for H₂S as an endogenous mediator stems beyond that of cardioprotection and the range of body systems and cell types is continually expanding. As H₂S appears to be characteristic of NO it may possess multiple mechanisms and sites of action. Identification of these will assist in revealing the physiological significance and potential for medical exploitation of this surprisingly complex gaseous mediator. The interaction of NO and H₂S also suggests a potential role for H₂S in erectile dysfunction which could lead to the discovery of additional therapeutic targets beyond the already successfully exploited PDE5 inhibitors. In addition H₂S production could also be targeted therapeutically to control hypertension. The ability to readily detect and measure H₂S the endogenous levels of H₂S in patients exhibiting various risk factors for progressive coronary artery disease may assist in identifying the role of H₂S in both disease progression and in cardioprotection. In this respect H₂S could act as a biomarker enabling the severity of coronary artery disease progression to be determined in patients and perhaps controlling it whilst in its infancy. Furthermore, understanding whether an imbalance in H₂S production is a causal factor or a consequence of coronary disease could be central for targeting the key metabolic pathways of H₂S production as a potential therapeutic strategy to inhibit coronary artery disease progression. With the emergence of improved pharmacological tools and the increased interest in H₂S, the next few years should provide a greater understanding of the physiological role of H₂S.

Current findings from World Health Organisation predict that as far as 2030, cardiovascular disease will remain the leading cause of mortality in the world (Mathers *et al.*, 2006), there is a need for more effective solutions to protect patients suffering from acute myocardial infarction. Due to the unpredictable nature of the onset of acute myocardial infarction, clinical therapies should ideally be aimed at the time of reperfusion when flow is restored to the heart and minimise reperfusion

injury. The recent discovery of the RISK pathway, and its expanding components and activators, provides the basis for a greater understanding of the cardioprotective mechanisms that occur at reperfusion and various algorithms that can be used to prime this pathway, therefore suggesting a novel target for cardioprotection. The increased interest in H₂S over the past decade has led to its discovery in the myocardium and also revealed the potential cardioprotective effect of exogenous H₂S, suggesting a potential endogenous mediator of cardioprotection. However, there is only a weak characterisation of the enzymes that synthesise H₂S in the myocardium and the effect of ischaemia-reperfusion on their ability to function. As yet a pharmacological target for H₂S has not been identified or a specific mechanism of action determined.

Major points of focus:

The aim of the studies described in this thesis was to characterise H₂S synthesis in the myocardium and elucidate potential cardioprotective roles of H₂S during ischaemia-reperfusion.

The general hypothesis underpinning this work was that H₂S synthesising enzymes exist in the myocardium and the resulting H₂S provides cardioprotection against ischaemia-reperfusion.

The specific questions addressed are:

1. Does the rat myocardium contain H₂S synthesising enzymes and is there a predominant isoform?
2. What effect does ischaemia-reperfusion have on the ability of the rat myocardium to synthesise H₂S?
3. Does exogenous or endogenously stimulated H₂S protect the rat myocardium against regional ischaemia-reperfusion injury?
4. Does H₂S activate the RISK pathway to induce cardioprotection in the rat myocardium?

The approach to these scientific questions involved using a variety of experimental techniques and rat myocardium. The techniques undertaken included PCR and Western blotting to identify the presence of the mRNA and protein for the H₂S synthesising enzymes in rat myocardium (Chapter 3). Cloning of the H₂S synthesising enzymes and transfection into cell lines, to validate the specificity of a custom synthesised antibody to CSE used for Western blotting studies. Isolated perfused rat hearts were used to investigate the effect of regional ischaemia-reperfusion and observe the effects of exogenous and endogenously stimulated H₂S on infarct size limitation (Chapter 4). The isolated perfused hearts were also used to decipher the mechanism of cardioprotection elicited by H₂S by use of pharmacological tools to inhibit components of the RISK pathway (Chapter 6). Biochemical assays were also performed to measure the tissue concentrations of H₂S and also determine the maximal H₂S synthesising capacity of exogenously stimulated enzyme and observe the effect of ischaemia and reperfusion (Chapter 5).

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Appendix 1: CSE cloning and transfection methods**Generation of a CSE expression vector**

In an attempt to validate the protein expression studies of the CSE enzyme in rat tissues, a series of experiments were performed to construct a CSE expression vector for use in transient transfection analyses. The CSE construct was kindly generated by Dr. Steve Allen (RVC). The full length open reading frame (ORF) of the mouse CSE gene was generated by splicing together two Image clones (from Geneservice Ltd., Cambridge, UK) that overlapped an EcoR1 site present in the sequence. Specifically the full length ORF was generated by digesting both clones with EcoR1 and gel purifying the fragment from each digest that contained the CSE ORF. These fragments consisted of the 3' ORF inserted into the plasmid pExpress1 and the 5' ORF from the internal EcoR1 to an EcoR1 in the multi cloning site immediately upstream of the start ATG. These two fragments were then ligated to generate the full length ORF. The resulting expression vector was subjected to restriction enzyme digest to confirm the correct orientation of the 5' portion of the CSE gene and a maxi-prep plasmid preparation was performed to generate sufficient plasmid for transfection studies.

Transient expression of CSE in rat GH3 somatolactotroph cells

The GH3 cell line was chosen as a heterologous expression model in which to examine the expression of CSE, due to its availability and the fact that GH3 cells are of rat origin. Briefly, 0 to 2.5 µg/well of CSE plasmid was transfected in to GH3 cells plated at a density of 10^6 cells/well. Total protein extracts were produced 48 h post transfection, and these cell lysates were subjected to Western blotting for CSE, as described in section 3.6.

Appendix 2: Summary of H₂S production assay validation studies

The H₂S production assay was adapted from methods published in the literature (Stipanuk *et al.*, 1982) to enable the production of H₂S to be measured in cardiac and liver tissue. Liver was used to establish the assay because it is a rich source of both the H₂S producing enzymes CSE and CBS, and because the tissue was in abundant supply. Initially, substrate concentrations were taken from the literature and initially L-cysteine 2 mM and P-5'-P 0.05 mM were tested. The H₂S yield was found to be very low and difficult to accurately detect using the spectrophotometer (as shown in figure 1). The majority of more recently published papers used higher concentrations of substrate and enzyme. L-cysteine 10 mM and P-5'-P 2 mM was tested and found to give higher absorbance values, indicating a higher yield of H₂S (as shown in figure 1). A 10% tissue homogenate was also found to be sufficient for the assay and meant less tissue could be used and the tissue was more easily re-suspended and pipetted without causing tip blockage as found with higher concentrations of tissue (20 and 40% w/v). The thickness of filter paper onto which the gas was absorbed was also investigated. Thick filter paper (0.34 mm) was found to be more effective at absorbing the H₂S gas produced than thinner paper (0.1 mm) (as shown in figure 1).

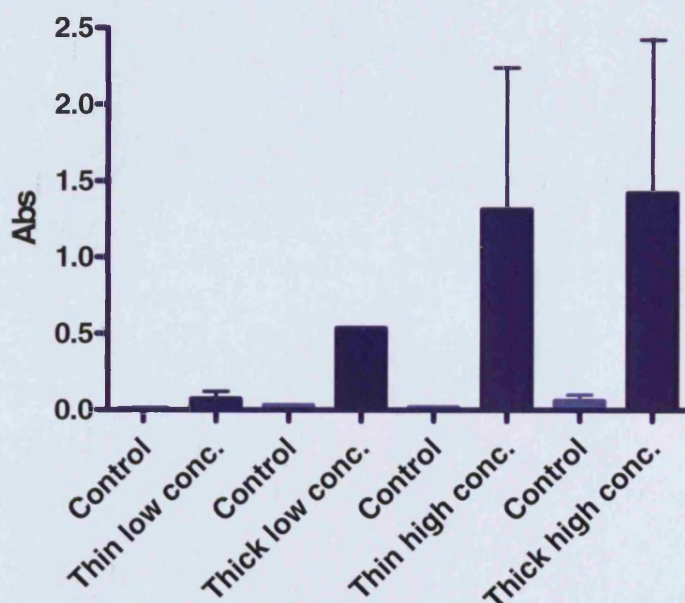


Figure 1 Preliminary absorbance results from the H_2S production assay, comparing the effects of low and high concentrations of L-cysteine (2 mM vs. 10 mM) and Pyridoxal-5'-phosphate (50 μM vs. 2 mM), and the effect of thin (0.34mm) and thick filter paper (0.1 mm). Abs= absorbance value. Each value shown is the mean \pm SEM from 2 separate experiments.

Initially problems were encountered with variability of results between experiments. This was especially the case with the NaHS standard curve, despite using the same aliquots on the same day, variation and reduction in absorbance levels occurred. NaHS, the H_2S donor was found to be unstable when stored in the freezer at -20°C ; as a result fresh results were prepared on each day of experiment. Fresh stocks of NaHS were also prepared prior to performing a second experiment on the same day. The effect of storing NaHS samples in the freezer is shown in figure 2, where the same stocks produced up to a 3 fold decrease in absorbance when used 3 days later, suggesting instability of the H_2S donor particularly at the higher concentrations where stored at -20°C .

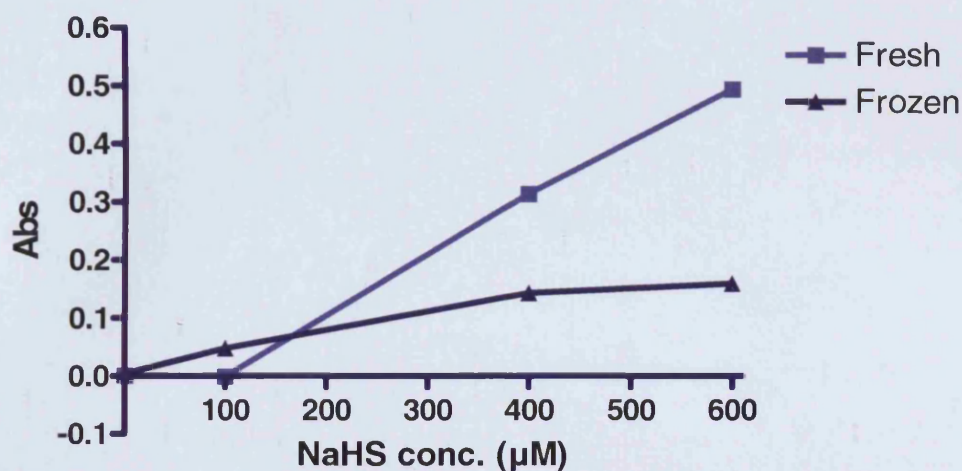


Figure 2 Comparison of the standard curves to freshly prepared and frozen (3 days, -20°C ,) stocks of NaHS. Abs= absorbance value. ($n=1$).

A reduction in the absorbance values produced by the NaHS was observed between experiments. Solid NaHS had started to absorb moisture and is hygroscopic and therefore when a fixed amount was weighed out to prepare the stocks it did not contain as much NaHS as was calculated for a dry stock of the compound. The solid stock of NaHS was replaced and far greater absorbance values were produced at all the NaHS concentrations (as shown in figure 3). As a result of these findings the solid NaHS stock is to be replaced more regularly since this compound appears susceptible to absorbing moisture from the air.

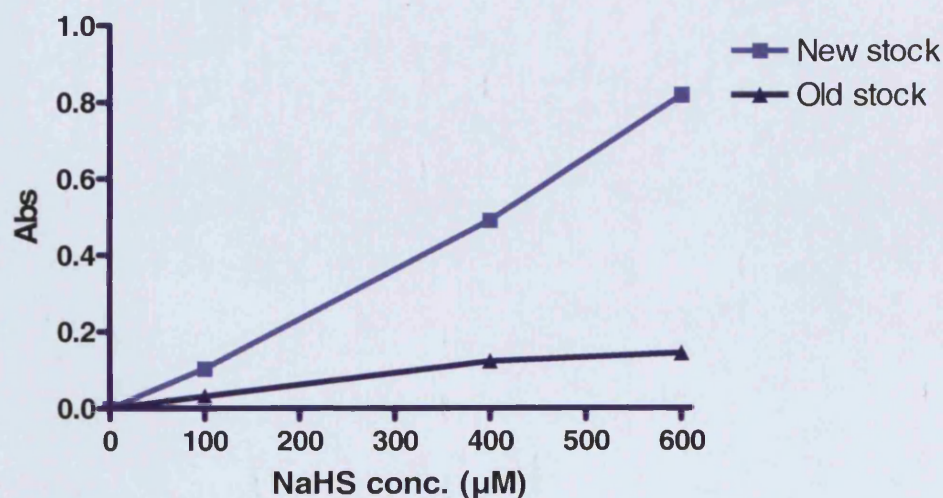


Figure 3 Comparison of the standard curves to NaHS with freshly prepared samples from a new solid stock of NaHS vs. a freshly prepared samples from an old solid stock of NaHS. Abs= absorbance value. ($n=1$).

Temperature was also found to be a significant parameter resulting in variability in the results between experiments for the tissue samples tested. A more thermostatically stable water bath was used and was also covered to create a more even temperature distribution over the reaction flasks. When tissue samples were tested in duplicate, there was very little variation between the samples.

After addressing each of these variables individually and investigating ways of improving them, a H₂S production assay was established that enables both myocardium and liver tissue H₂S production rates to be accurately and reproducibly measured.

Appendix 3: Preparation of assay solutions**PCR reagents (Chapter 3)****A PCR “master mix” was prepared per sample**

Buffer (10x)	2 µl
dNTP's	1 µL
Taq polymerase	0.5 µl
Forward primer	1 µl
Reverse primer	1 µl
“Q” solution	4 µl
DEPC treated H ₂ O	5.5 µl
 Total volume per sample	 15 µl

Loading dye

Bromophenol blue	10 mg
Xylene cyenol	10 mg
Sterile glycerol	1.2 ml
10x TBE	0.8 ml
d.H ₂ O	2.0 ml

10x TBE stock solution

Tris	30.3 g
Boric acid	15.5 g
EDTA (disodium)	1.9 g

Dissolve in 500 ml d.H₂O pH 8.0

1.6% agarose gel

Agarose	8 g
10x TBE d.H ₂ O	50 ml

The solution was then heated in the microwave for 3 minutes in order to dissolve and aliquoted into 50 ml tubes. When the gels were required, a 50 ml aliquot was melted in the microwave for 90 seconds and once dissolved 5 µl of ethidium bromide 10 mg/ml was added and mixed into the melted gel. The gel was cast in a mould and allowed to set for at least 20 minutes prior to using.

Western blotting reagents (Chapter 3)

Lysis buffer for protein extraction (250 ml)

20 mM Tris-HCl: pH 7.6

10 mM EDTA: pH 8.0 0.93 g

0.25% Triton X-100 625 µl

100 mM NaCl 1.46 g

10 mM KCl 0.19 g

3 mM MgCl₂.6H₂O 0.15 g

Make up 250 mM Tris-HCl, pH 7.6 with 1 M HCl

Make up 250 mM EDTA, pH 8.0 with 1 M NaOH

Add 10 ml EDTA (10 ml DETA in 250 ml = 10 mM)

Add 20 ml Tris-HCl (20 ml Tris-HCl in 250 ml = 20 mM)

200 µl aliquots were prepared and 10 µl of protease inhibitor cocktail (Sigma-Aldrich, UK) was added to each

Aliquots were stored at -20 °C until required

4% stacking gel (makes 2 mini gels)

d.H₂O 3.4 ml

30% protogel 850 µl

1.5 M Tris-HCl pH 6.8 630 µl

50 µl SDS 50 µl

10% AMPS (0.2 g/2ml) 50 µl

TEMED 5 µl

10% resolving gel

d.H ₂ O	6.9 ml
30% protogel	4.0 ml
1.5M Tris-HCl pH 8.4	3.8 ml
10% SDS	150 µl
AMPS (0.2g/2ml)	150 µl
TEMED	9 µl

Running buffer (10x) (1L)

0.25 M Tris-base	30.3 g
1.92 M Glycine	144 g
1% (w/v) SDS	10g

Make up to 1x solution using d.H₂O and pH to 8.8

Transfer buffer (1L)

2.5 mM Tris Base	3.03 g
0.2 M Glycine	14.4 g
20% MeOH	200 ml

d.H₂O to make 1 L

pH 8.5 in d.H₂O

TBS (x10) (1L)

0.2 M Tris base	24.2 g
1.37 M NaCl	80 g

pH 7.6 with HCl

TBST

Dilute 10x TBS 1:10 in d.H₂O

Add 0.1% Tween (1 ml per 1 L)

CSE protein purification using protein G HP purification column (Chapter 3)

Binding buffer

20 mM sodium-phosphate, pH 7.0

Elution buffer

0.1 M glycine-HCl, pH 2.7

Eluted fractions were neutralised in 1 M Tris-HCl, pH 7.0

Langendorff isolated perfused heart experiments (Chapters 4, 5, 6)

0.5% Evans blue solution

0.5 g of Evans Blue powder in 100ml of d.H₂O

1% triphenyltetrazolium solution

1 g of chloride salt in 100 ml of phosphate buffered saline

H₂S production assay reagents (Chapter 5)

Sodium phosphate buffer (pH 7.4)

100 mM stocks of NaH₂PO₄ and Na₂HPO₄ were prepared in d.H₂O

They were combined 2 parts NaH₂PO₄ and 8 parts Na₂HPO₄ to produce a pH 7.4 buffer

