Role of endogenous hydrogen sulfide synthesised by cystathionine-gamma-lyase in the inflammatory response in acute pancreatitis

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Abstract

Mammals have the ability to synthesise endogenous hydrogen sulfide via desulphhydration enzymes though the physiological effects are still relatively unclear particularly in the inflammatory response. Endogenous hydrogen sulfide has been shown to be both pro and anti-inflammatory in numerous models of disease in animals. Common aspects of these studies are the reported dysregulation of tissue cystathionine \( \gamma \)-lyase (CSE) expression (the major hydrogen sulfide producing enzyme in the periphery) and the use of \( \text{D,L-} \) propargylglycine (PAG) to inhibit CSE activity. However, PAG is not a specific inhibitor and very few studies have correlated CSE dysregulation with changes in endogenous tissue hydrogen sulfide levels. Therefore the aims of this thesis were to investigate pancreatic and macrophage CSE dysregulation in acute pancreatitis, to correlate this dysregulation with changes in endogenous hydrogen sulfide levels/ hydrogen sulfide modified proteins and to determine the effect of CSE gene deletion on disease severity and the inflammatory response. Results in this thesis show that acute pancreatitis directly mediated upregulation in pancreatic CSE expression which was a gap in the existing body of knowledge. I have also found novel evidence of acute pancreatitis induced upregulation of CSE in macrophages with \textit{in vitro} studies using plasma from pancreatitic mice and elucidated the molecular mechanisms involved. CSE gene deletion resulted in an amelioration of acute pancreatitis with reduced plasma amylase levels, pancreatic and lung neutrophil infiltration, oedema as well as acinar cell damage. There was also a reduction in pancreatic pro-inflammatory mediators, MCP-1, MIP-2\( \alpha \), IL-6, and PGE\(_2\) as well as NF\(\kappa\)B activation. Similar findings were found \textit{in vitro} using isolated primary pancreatic acini. The results of this thesis incontrovertibly show that CSE exerts pro-inflammatory effects in acute pancreatitis and corroborates with previous studies using PAG. It also shows upregulation of CSE in stimulated macrophages thus suggesting a role for endogenous hydrogen sulfide in the modulation of macrophage activation.
Acknowledgments

To my family for your unconditional love

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To my friends, far and wide, who kept me sane and going no matter what

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To Cikgu Siti Rokiah binti Ahmad for nurturing the passion and belief
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<td>CBS</td>
<td>Cystathoinine-(\beta)-synthase</td>
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<tr>
<td>3MST</td>
<td>3-mercaptopyruvate sulfurtransferase</td>
</tr>
<tr>
<td>CAT</td>
<td>Cysteine aminotransferase</td>
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<td>TRPV1</td>
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<td>Interleukin 6</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemotactic protein 1</td>
</tr>
<tr>
<td>MIP-2(\alpha)</td>
<td>Macrophage inflammatory protein 2 (\alpha)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>SIRS</td>
<td>Systemic inflammatory response syndrome</td>
</tr>
<tr>
<td>MODS</td>
<td>Multiple organ damage syndrome</td>
</tr>
<tr>
<td>RAS</td>
<td>Renin-angiotensin system</td>
</tr>
<tr>
<td>SP</td>
<td>Substance P</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>AP</td>
<td>Acute pancreatitis</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>ox-LDL</td>
<td>Oxidised low density lipoprotein</td>
</tr>
<tr>
<td>ZnS</td>
<td>Zinc sulfide</td>
</tr>
<tr>
<td>PAAF</td>
<td>Pancreatitis associated ascitic fluid</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>HPRT</td>
<td>Hypoxanthine-guanine phosphoribosyltransferase</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3′-Diaminobenzidine</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>biotin-HPDP</td>
<td>N-(6-(biotinamido)hexyl)-3′-(2′-pyridyldithio)-propionamide</td>
</tr>
<tr>
<td>MMTS</td>
<td>S-Methyl methanethiosulfonate</td>
</tr>
<tr>
<td>MSBT</td>
<td>Methylsulfonyl benzothiazole</td>
</tr>
<tr>
<td>CN-biotin</td>
<td>Cyanoacetate-biotin</td>
</tr>
<tr>
<td>sp1</td>
<td>Specificity protein 1</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-4,5-bisphosphate 3-kinase</td>
</tr>
<tr>
<td>Akt</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>GSK3</td>
<td>Glycogen synthase kinase 3</td>
</tr>
<tr>
<td>NK-1R</td>
<td>Neurokinin-1 receptor</td>
</tr>
<tr>
<td>PGE₂</td>
<td>Prostaglandin E₂</td>
</tr>
<tr>
<td>LTB₄</td>
<td>Leukotriene B₄</td>
</tr>
<tr>
<td>HMGB1</td>
<td>High mobility group box 1</td>
</tr>
</tbody>
</table>
1 Introduction

Although essential in a host’s defense against infections and injuries, inflammation itself contributes to the pathophysiology of many diseases (1) ranging from chronic conditions such as arthritis to acute conditions such as sepsis. It is also thought to be a contributing factor in the development of cancer (2, 3). It was touted as the secret killer by the Times magazine (4) and the socioeconomic cost and burden of all these inflammatory related diseases combined would be enormous. A better understanding of the inflammatory response has brought about advances in treating diseases over the years, however there is still much to be understood especially in light of new players to the game. Hydrogen sulfide is one such new player. In this chapter, I will introduce the various sources of endogenous hydrogen sulfide, its reported physiological roles and the proposed methods by which it exerts its effect. I will then relate these findings to the inflammatory response with a focus on acute pancreatitis followed by the aims of this thesis.

1.1 Hydrogen sulfide

Hydrogen sulfide is a colourless gas that has the characteristic odour of rotten eggs. It is readily soluble in water (~80 mM at 37°C (5)) to form an equilibrium between the species shown below along with reported dissociation constants (5, 6):

\[
\begin{align*}
\text{H}_2\text{S} & \rightleftharpoons \text{H}^+ + \text{HS}^- \\
6.8 \text{ at 37}^\circ\text{C} & \\
\text{pK}_{a1} & \\
\text{H}^+ + \text{HS}^- & \rightleftharpoons \text{H}^+ + \text{S}^{2-} \\
12 \text{ at 37}^\circ\text{C} & \\
\text{pK}_{a2} & 
\end{align*}
\]

Environmental sources of atmospheric hydrogen sulfide include biogenic sources of marine as well as terrestrial origins, natural sources such as volcanic and anthropogenic sources such as biomass burning (7). Hydrogen sulfide has long been known for its toxicity with the first description dating 300 years back (8). The effects of hydrogen sulfide toxicity is now well documented and range from mild pulmonary irritation to death (8). However it was not until the end of the 20th century that the perception of hydrogen sulfide was to be altered from that of a purely toxic molecule to one that possesses physiological relevance akin to oxygen, nitric oxide and carbon monoxide.
The discovery and purification of the major hydrogen sulfide synthesizing enzymes dates as far back as to 1958 (Cystathionine-\(\gamma\)-lyase, CSE) (9) and 1968 (Cystathionine-\(\beta\)-synthase, CBS) (10), however these enzymes were then better known for their roles in the transsulfuration pathway. The capacity for these enzymes to synthesize hydrogen sulfide \textit{in-vivo} by mammals was first demonstrated by Stipanuk et al (11) in 1982. However it took a little over 10 years after that for the landmark study by Abe et al (12) which was the first published report detailing the potential role of endogenous hydrogen sulfide as a neuromodulator. Since then, there has been an explosion of publications attempting to determine the possible physiological roles of hydrogen sulfide.

1.2 Endogenous hydrogen sulfide

Currently there are two known sources of endogenous hydrogen sulfide; the enzymatically derived source and the labile hydrogen sulfide source. The enzymatic source is mostly thought to be a byproduct of enzymes in the transsulfuration pathway. There is also an overlap between these two sources as the enzymatic driven synthesis of hydrogen sulfide is thought to fuel the generation of labile hydrogen sulfide that in turn serves as a readily available pool of hydrogen sulfide. This section will describe the nature and interplay of these two sources in more detail.

1.2.1 Enzymatic source

There are currently three known enzymes that are capable of synthesizing hydrogen sulfide; cystathionine-\(\gamma\)-lyase (CSE) (EC 4.4.1.1), cystathionine-\(\beta\)-synthase (CBS) (EC 4.2.1.22) and 3-mercaptopyruvate sulfurtransferase (3MST) (EC 2.8.1.2). Of these three enzymes both CSE and CBS are cytosolic while MST is found in the mitochondria (13). The tissue distributions of these three enzymes are varied in location as well as levels of expression. The liver and kidney are the two organs that express all three enzymes at high levels (14-16). CBS is also expressed in the brain, lung, stomach and pancreas (14) while CSE is found in the stomach, small intestine, pancreas and smooth muscle (16-18) and 3MST in the brain, heart, lung, thymus and testis (15). There is also varied expression levels of CSE and CBS in tissues where both enzymes are co-expressed; the liver and kidney express higher levels of CSE compared to CBS, 60 and 20-fold respectively (19).
CSE and CBS are enzymes involved in the transsulfuration pathway and are directly involved in the generation of hydrogen sulfide as a byproduct through their diverse catalyzed reactions (20). Under physiological conditions, the major reaction catalyzed by CSE in the generation of hydrogen sulfide is via the α,β-elimination of cysteine (21):

while the major reaction catalyzed by CBS in the generation of hydrogen sulfide is via the condensation of cysteine and homocysteine to produce cystathionine (20):

3MST on the other hand catalyzes the conversion of 3-mecaptopyruvate to pyruvate resulting in the formation of a 3MST persulfide. This persulfide moiety then requires specific reducing agents such as thioredoxin and dihydrolipoic acid to release hydrogen sulfide (22). Therefore it is perceived that the major contributors of endogenous hydrogen sulfide are CSE and CBS (19) although it has been shown that 3MST does contribute to endogenous hydrogen sulfide generation in certain tissues (23, 24). Figure 1.1 summarises the pathways of CSE, CBS and 3MST mediated hydrogen sulfide synthesis and its relation with the transsulfuration pathway.
Figure 1.1 Major endogenous hydrogen sulfide synthesising pathways. The transulfuration pathway involves the conversion of homocysteine to cysteine by CBS and CSE. These enzymes are also responsible for the majority of endogenous hydrogen sulfide synthesis through reactions shown in black.

The relative contribution of CSE and CBS to endogenous hydrogen sulfide synthesis is dependent on the expression levels of each enzyme, the concentration of their preferred substrates and the extent of CBS allosteric activation by S-adenosylmethionine (AdoMet) (19, 20). For example, both CSE and CBS were found to contribute equally to hydrogen sulfide synthesis at high substrate concentrations of cysteine and homocysteine in the liver; however at physiologically relevant substrate concentrations (100 μM cysteine and 10 μM homocysteine) and taking into account the ratio of CSE to CBS (60:1) present in the liver, the contribution of liver hydrogen sulfide synthesis by CSE was estimated to be around 97 % and CBS 3 % (19). Taken together, the differential enzymatic expression and substrate preference suggests that the contribution of each enzyme to endogenous hydrogen sulfide synthesis may have separate roles to play in the physiology of different cells and tissues.
1.2.2 Labile source

Apart from enzymatic synthesis of free hydrogen sulfide, there are bound sulfur sources that could release free hydrogen sulfide upon acidification or reduction of the parent molecule; these sources are termed acid-labile sulfide and DTT-labile sulfide (or sulfane sulfur) respectively (25). The relative amounts of free sulfide present in these discreet pools in biological samples are still a subject of continuous study as more sensitive and specific methods are continuously developed. At present, human plasma acid-labile sulfide is reported to be in the low micromolar range followed by sulfane sulfur and free sulfide, both of which are at similar low nanomolar concentrations (26). An initial study on tissue labile sulfide levels reported low nmol/g levels in several murine tissues; the brain was reported to contain 12.5 nmol/g and 18.5 nmol/g of acid labile and sulfane sulfur respectively (27). However much higher levels of bound sulfide were reported recently in tissue homogenates (28), as a comparison the mouse brain was shown to contain 161 nmol/g protein and 1481 nmol/g protein of acid-labile and sulfane sulfur respectively. These differences could be attributed to the different methods employed in each study; fluorescence based HPLC (27) and direct measurement with gas chromatography (28).

The major source of acid-labile sulfide are iron-sulfur cluster containing proteins (29), these proteins are ubiquitous, diverse and serve a wide array of functions (30). Although the iron-sulfur cluster containing proteins provides a substantial source of labile sulfide, it is an unlikely physiological endogenous source as a maximum of pH 5.4 is required to release the bound sulfur as sulfide (28).

Sources of sulfane sulfur include thiosulfate, thiosulfonates, polysulfides and protein persulfides (29). Although reducing agents are present in the cell (ie glutathione (GSH) and cysteine), it has been shown that the alkalization of the cytoplasm is required for effective release of hydrogen sulfide from these sulfane sulfur sources (28). This has been shown in rat astrocytes where high concentrations of extracellular K⁺ are normally present when nearby neurons are excited (28).

Among the available sources of sulfane sulfur, protein persulfides are particularly intriguing as they are not synthesized de novo as a persulfide but are a product of a cystiene thiol modification by the addition of divalent sulfide molecule resulting in the formation of an R-S-SH; this process is termed s-sulfhydration akin to s-nitrosylation (31). A large number of proteins have been reported to be basally s-sulfhydrated and the addition of exogenous hydrogen sulfide further increases the level of s-
sulphydration (31) (discussed further in section 1.4.2). Hydrogen sulfide has also been shown to be scavenged in proteinaceous biological solutions such as plasma (32), tissue homogenates (28, 33) and single protein solutions containing cysteine residues (32). These scavenged hydrogen sulfide was then partially retrieved by applying a reducing agent to the homogenates (28, 33). This suggests that protein persulfides could serve as both a source as well as a sink of endogenous free hydrogen sulfide.

**Figure 1.2 Endogenous source of hydrogen sulfide in mammals.** Endogenous source of free hydrogen sulfide can be derived from two sources, enzymatic and labile sulfide. The enzymatic source of hydrogen sulfide involves the actions of the transulfuration pathway enzymes; cystathionine-γ-lyase (CSE) (EC 4.4.1.1), cystathionine-β-synthase (CBS) (EC 4.2.1.22) and 3-mercaptopyruvate sulfurtransferase (3MST) (EC 2.8.1.2). Both CSE and CBS are able to catalyse the synthesis of hydrogen sulfide through multiple reactions, however L-cysteine and L-cysteine + homocysteine are the preferred substrates for CSE and CBS respectively under physiological conditions (20, 21). The generation of hydrogen sulfide by 3MST is not direct and utilises 3-mercaptopyruvate as its substrate (generated by cysteine aminotransferase (CAT) from L-cysteine). 3MST catalyses the conversion of 3-mercaptopyruvate to pyruvate resulting in the generation of a persulfide moiety on its cysteine residue, the reduction of this persulfide yields free hydrogen sulfide (22). Acid labile sulfide is a source of hydrogen sulfide that is liberated under acidic conditions, a major source are the iron-sulfur clusters of non-heme iron-sulfur proteins. Sulfane sulfur atoms are defined as divalent sulfur bonded only to other sulfur and is released as hydrogen sulfide upon reduction. Sulfane sulfur containing molecules such as protein persulfides are thought to be generated from the interaction of hydrogen sulfide and cysteine residues of proteins (31) and could therefore serve as a potential store and source of endogenous hydrogen sulfide (28).
1.2.3  **Endogenous levels of hydrogen sulfide**

There is still very little consensus as to the levels of endogenous free hydrogen sulfide. This is due to the difficulty in accurately measuring hydrogen sulfide in a biological solution. Among the issues associated with measuring hydrogen sulfide is its volatility and difficulty in distinguishing free sulfide from labile sulfide. Hydrogen sulfide in its gaseous form readily diffuses out of solution resulting in loss of hydrogen sulfide, this makes sample preparation complicated particularly from tissues that require some form of homogenization step. As mentioned in section 1.2.2, there is a readily available source of labile hydrogen sulfide that could be released upon acidification or reduction, certain detection methods that employ such conditions may overestimate actual levels of free hydrogen sulfide, this include the methylene blue method (11) that was widely used in the initial hydrogen sulfide studies. The methylene blue method is a spectrophotometric approach involving the incorporation of sulfide into methylene blue in the presence of acidic N,N-dimethyl-p-phenylenediamine and ferric chloride as the oxidising agent (34) (further elaborated in section 1.2.4). Initial reports that used the direct spectrophotometric methylene blue method of measuring free hydrogen sulfide showed high micromolar hydrogen sulfide levels of between 30 to 300 µM in circulation (32) and in tissues (35). More recent methods however have shown low or sub nanomolar concentrations of circulating hydrogen sulfide (36) and pmol per g levels in tissue (37). These estimations were made by direct measurement using gas chromatography (35, 37) as well as an indirect hydrogen sulfide derivatized and separation method using monobromobimane as the derivatizing agent followed by separation and detection by HPLC (36). The reason for the big divide has been thought to be due to the harsh acidic nature of the methylene blue assay that could potentially liberate acid labile sulfide from the biological matrices (25, 29, 37). However, despite the improvement of recent methods over the direct spectrophotometric methylene blue method, it has recently been argued that even the reported low micromolar levels of free sulfide in plasma (36) and pmole per g levels in tissue (37) should yield a detectable odour by the human nose which it does not (38). Therefore, it is still unclear as to what the actual levels of free sulfide are in circulation and in tissues.
<table>
<thead>
<tr>
<th>Method</th>
<th>Species</th>
<th>Organ</th>
<th>Free hydrogen sulfide</th>
<th>Acid-labile hydrogen sulfide</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gas chromatography/flame photometric or chemiluminescence</td>
<td>Rat</td>
<td>Liver</td>
<td>ND</td>
<td>112 nmol/ g</td>
<td>(39)</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>Heart</td>
<td>ND</td>
<td>274 nmol/ g</td>
<td>(39)</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>Liver</td>
<td>17 nM (~17 pmol/ g)</td>
<td>~150 nmol/ g</td>
<td>(37)</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>Brain</td>
<td>14 nM (~14 pmol/ g)</td>
<td>~300 nmol/ g</td>
<td>(37)</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>Heart</td>
<td>&lt; 0.1 nmol/ g</td>
<td>~90 nmol/ g</td>
<td>(37)</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>Heart</td>
<td>6 umol/ g</td>
<td>~175 nmol/ g prot</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ion chromatography</td>
<td>Rat</td>
<td>Brain</td>
<td>46 nmol/ g</td>
<td></td>
<td>(42)</td>
</tr>
<tr>
<td>Methylene blue using RP-HPLC</td>
<td>Rat</td>
<td>Liver</td>
<td>145 nmol/ g</td>
<td></td>
<td>(43)</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>Brain</td>
<td>69 nmol/ g</td>
<td></td>
<td>(43)</td>
</tr>
<tr>
<td>Methylene blue assay using spectrophotometry</td>
<td>Mouse</td>
<td>Plasma</td>
<td>~35 μM</td>
<td></td>
<td>(44)</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>Plasma</td>
<td>~50 μM</td>
<td></td>
<td>(45)</td>
</tr>
<tr>
<td>Monobromobimane derivatisation/ RP HPLC</td>
<td>Mouse</td>
<td>Plasma</td>
<td>~0.8 μM</td>
<td>~ 3.5 μM</td>
<td>(26)</td>
</tr>
<tr>
<td></td>
<td>Plasma</td>
<td></td>
<td>0.2 – 0.3 μM</td>
<td></td>
<td>(46)</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td></td>
<td>~175 nmol/ g prot</td>
<td></td>
<td>(47)</td>
</tr>
<tr>
<td>Fluorescent probes</td>
<td>Mouse</td>
<td>Plasma</td>
<td>56 μM</td>
<td></td>
<td>(48)</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>Blood</td>
<td>32 μM</td>
<td></td>
<td>(49)</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>Brain</td>
<td>~1.7 μmol/ g prot</td>
<td></td>
<td>(50)</td>
</tr>
</tbody>
</table>

Table 1 Reported levels of endogenous free and acid-labile hydrogen sulfide content in tissues and plasma. This table shows current reported endogenous hydrogen sulfide levels as measured by various methods of detection. ND = not detectable, g = wet weight of tissue.
1.2.4 Methods of detecting free hydrogen sulfide

The detection of free hydrogen sulfide is complicated due to the gaseous nature and chemistry of the molecule. As a gaseous molecule there is the issue of passive loss due to volatility. However the proportion of hydrogen sulfide that exists as either the deprotonated gaseous form (\(H_2S\)), monoanion (\(HS^-\)) or dianion (\(S^{2-}\) ) species is dependent on the pH of the environment according to the reported dissociation constants (5, 6):

\[
H_2S \overset{pK_{a1}}{\rightleftharpoons} H^+ + HS^- \overset{pK_{a2}}{\rightleftharpoons} H^+ + S^{2-}
\]

Since there are only very rare situations where the internal pH could reach pH 12, the predominant species of physiological hydrogen sulfide would exist as either the gaseous or monoanion form. At pH 7.4 and 37°C, ~20% of sulfide exists as hydrogen sulfide gas, while at pH 7.4 and 25°C ~30% of sulfide exists as hydrogen sulfide gas. Therefore methods of measuring endogenous free hydrogen sulfide should be able to detect both species (\(H_2S\) and \(HS^-\)) as well as take into account possible loss of the gaseous form due to volatility, all of which are tightly linked to the pH of the solution. The reactivity of dissociated \(HS^-\) is high, as it is more readily oxidized than \(H_2S\) (51) thus adding another layer of complexity as the less volatile \(HS^-\) species though readily soluble in aqueous solutions is susceptible to oxidation by dissolved oxygen within buffers (52). The most commonly observed products and intermediates are polysulfides (\(S^{n-}; 2 < n < 5\) ), thiosulfate (\(S_2O_3^{2-}\) ), sulfite (\(SO_3^{2-}\) ), and sulfate (\(SO_4^{2-}\) ) (51) as shown below:

\[
\begin{align*}
2HS^- + O_2 &\rightarrow 2S^0 + 2OH^- \\
2HS^- + 2O_2 &\rightarrow S_2O_3^{2-} + H_2O \\
2HS^- + 3O_2 &\rightarrow 2SO_3^{2-} + 2H^+ \\
2HS^- + 4O_2 &\rightarrow 2SO_4^{2-} + 2H^+
\end{align*}
\]

Therefore attempts to detect and measure free hydrogen sulfide in biological material should take into account factors such as volatility of the hydrogen sulfide species, effect of pH on sulfide dissociation, oxidation of free sulfide, selectivity of the probe/detection towards both \(HS^-\) and \(H_2S\) species as well as possible liberation of bound sulfide (discussed in section 1.2.2) due to assay conditions. To date,
there have been several methods developed to detect and measure endogenous levels of hydrogen sulfide. These detection methods for sulfide have evolved from simple spectrophotometric assays to the more recently used techniques such as high pressure liquid chromatography, gas chromatography, and polarographic, electrochemical and chemiluminescent detection that are highly specific and sensitive with much lower detection limits. However, each technique has certain advantages as well as limitations in the attempt to detect and measure bioavailable free hydrogen sulfide. These methods can be generally broken down into two categories which involve either direct detection of free sulfide or prior derivatisation. Direct detection of sulfide can be made in real-time using electrochemical polarographic sensors and as an end point measurement with fluorescent probes and gas chromatography. Electrochemical polarographic sensors developed by Doeller et al are made up of an anode, cathode and electrolyte assembly protected from solution constituents by H2S permeable polymer sleeves (53, 54). As such, these sensors are specific but only capable of detecting the gaseous hydrogen sulfide species and have been utilised to detect hydrogen sulfide production by cells and intact tissue incubated with L-cysteine as the hydrogen sulfide enzyme producing substrate (53-55), but has not yet been used to measure hydrogen sulfide content in biological fluids or tissues. An array of fluorescent probes have also been developed to detect and measure hydrogen sulfide. The general concept of these probes revolve around modification of a fluorophore resulting in quenching. These modified fluorophores are designed to be sensors and upon reacting with hydrogen sulfide restores the fluorescent properties of the fluorophore. There are three methods of modification; alteration of an amine to an azide or nitroso group at a favourable position which would render it more susceptible to reduction by hydrogen sulfide over other reductants (56-59), conjugation with a dicopylamine or an azamacrocyclic group to promote Cu²⁺ coordination and quenching which would then react with sulfide ion to form CuS thus removing the Cu²⁺ ion (60, 61), and finally conjugation with a hydrogen sulfide specific Michael acceptor which involves two steps of first reduction of the most electrophilic component to form a free thiol followed by a Michael addition reaction that would either cleave off a quencher or to complete the fluorophore structure (62, 63). Coupled with techniques such as two photon microscopy and fluorescence resonance energy transfer, the lower limit of detection of hydrogen sulfide using fluorescent sensors have been reported to be in the low nanomolar region (64, 65). However despite the improvements in selectivity and sensitivity of these probes, there is very little
demonstration of its ability to quantify endogenous levels of hydrogen sulfide with only three reports so far in plasma and brain tissue of mice (48-50). There is also the issue of specificity; in reports using the reducing ability of hydrogen sulfide as the sensing method, hydrogen sulfide has been shown to be more reactive over other thiol species such as reduced glutathione and cysteines by about 100x at best. However the intracellular glutathione levels are in the order of ~10 mM and for the sensors to be specific the levels of hydrogen sulfide needs to be within the 0.1 mM to 10 mM range which is unlikely given the current perceived levels of endogenous hydrogen sulfide. Another direct method of measuring hydrogen sulfide is by gas chromatography which offers the most sensitive option so far with lower detection limits in the pM range. Most reports that utilise gas chromatography to measure free tissue hydrogen sulfide employ a chemiluminescent sulfur detector (35, 37, 40, 41) and among them Levitt et al 2011 describes the most detailed method that takes into account the volatility of the gaseous hydrogen sulfide species during sample preparation and potential release of acid labile sulfide during measurement (37). This method involves homogenisation of tissues at pH 9.3 to ensure the stability of hydrogen sulfide by shifting the equilibria to the soluble anion species (HS\(^-\)), the pH is then lowered to ~5.8 to shift the equilibria to the gaseous species (H\(_2\)S) under air tight conditions to evolve free hydrogen sulfide out of solution and into the headspace which is then subsequently sampled and detected. Levitt et al 2011 reported very low levels of free tissue hydrogen sulfide in various murine tissues that were less than 0.1 nmol per g of wet weight including heart tissue. In contrast there were two other publications that reported 6 and 25 µmol per g of wet weight in murine heart tissue using the similar method described by Levitt et al 2011 with the exception of homogenisation in PBS at pH 7.4, which should in principal result in lower detection levels. The methods of derivatising hydrogen sulfide prior to detection have the benefit of trapping and stabilising free hydrogen sulfide prior to detection. The spectrophotometric approach utilising methylene blue as the colorimetric sensing dye (methylene blue method) has been the earliest and most widely used method used to measure endogenous levels of hydrogen sulfide down to low micromolar levels (12, 18, 42, 44, 66-69). In the methylene blue method, plasma or tissue homogenates (typically prepared at physiological pH) are reacted with zinc to fix free hydrogen sulfide through the formation of an insoluble ZnS precipitate. Subsequent reaction with N,N-dimethyl-p-
phenylene diamine under acidic conditions liberates $S^2$ from ZnS and reacts with the diarnines to form methylene blue in the presence of an oxidising agent, usually ferric ion (34):

The optical density of the resulting methylene blue (coefficient constant of 91,500 mol$^{-1}$ cm$^{-1}$ (70)) is measured at 670 nm wave length and the corresponding quantity of sulfide is read from a previously prepared calibration curve using sulfide salts. The entire assay is carried in a tube making it simple, however this also raises the issue of possible acid labile sulfide release from the biological matrix thus confounding the measurement with artifactually high results (25). Another criticism of the methylene blue is that the absorbance measured at 667 nm does not obey Beer’s law, however it was shown that this can be counteracted with the use of an appropriate ratio of N,N-dimethyl-p-phenylenediamine and iron(III)chloride to sulfide to achieve an optimal stoichiometry (71).

Derivatisation of hydrogen sulfide with monobromobimane under alkaline conditions (pH 9.3) to a stable and fluorescent sulfide dibimane product forms the basis of another method of detection developed by Shen at al (36):

This method has been used to measure free hydrogen sulfide in plasma through detection of the fluorescent sulfide dibimane product using reversed phase – high performance liquid chromatography for increased specificity and is capable of detecting hydrogen sulfide down to low nanomolar levels (36). This method has been mainly employed in measuring plasma levels (46, 72-76) with only one instance of use with liver tissue (47). A point to note is that the specificity of monobromobimane towards hydrogen sulfide and the ability to resolve between sulfide dibimane and other fractions have
come into question following a report attempting to compare spiked sulfide samples in buffer, plasma and whole blood using the monobromobimane as well as polarographic sensor method (38).

Figure 1.3 Recent methods developed to detect and measure endogenous levels of free hydrogen sulfide. This table summarises the current methods that have been developed as well as those that are currently used to detect and measure free hydrogen sulfide in cells, plasma and/or tissues. The polarographic sensor method was developed by Doeller et al (53); there are multiple probes designed to detect hydrogen sulfide (mentioned in text); the most practical and comprehensive method of gas chromatography method was developed by Levitt et al (37); the monobromobimane derivatisation/ RP-HPLC method was developed by Shen et al (36); and the methylene blue/ spectrophotometry method was first adopted in measuring endogenous hydrogen sulfide by Warencyia et al (42).

1.3 Physiological effects of hydrogen sulfide

The first published report detailing a direct physiological role of hydrogen sulfide was with regards to neuromodulation (12). Hydrogen sulfide has been shown to regulate neurotransmission by facilitating
the induction of hippocampal long term potentiation via selective enhancement of the NMDA receptor mediated responses through cyclic AMP; this was demonstrated by treating primary brain cells with exogenous hydrogen sulfide (1-100μM NaHS) (12, 77). Following this finding, hydrogen sulfide (0.1 to 10 mM NaHS) was also reported to modulate hypothalamo-pituitary-adrenal axis function by reducing potassium induced corticotropin-releasing hormone (CRH) release in rat hypothalamic explants (78). Endogenous hydrogen sulfide (using CBS and CSE inhibitors) and exogenous hydrogen sulfide (0.2-2.5 mM NaHS) have also been reported to elicit excitatory action in enteric neurons involving transient receptor potential vanilloid 1 (TRPV-1) receptors on extrinsic afferent terminals leading to increased chloride secretion in human and guinea-pig colonic submucosa/mucosa preparations(79).

By far the most widely studied role of hydrogen sulfide is its ability to induce smooth muscle relaxation and its implication in cardiovascular related conditions and diseases. This was first demonstrated by Hosoki et al (80) using exogenous hydrogen sulfide (NaHS) on pre-contracted ileum, portal vein and thoracic aorta of experimental animals with reported EC50 values of 180 μM, 160 μM and >1 mM respectively. A subsequent report by Zhao et al (18) further demonstrated that the hydrogen sulfide induced smooth muscle relaxation effect was mediated via the opening of KATP channels at NaHS/hydrogen sulfide concentrations of > 100 μM using aortic rings. These initial findings have been further corroborated and extended to other smooth muscle containing tissues involving the airways (81-83), gastrointestinal tract (84-86) and the cavernous (84, 87, 88). These studies have used both hydrogen sulfide synthesising enzyme inhibitors as well as a variety of exogenous hydrogen sulfide donors such as NaHS, Na2S and GYY4137 (morpholin-4-ium 4 methoxyphenyl(morpholino) phosphinodithioate) with effective concentrations ranging from 100 μM to 10mM.

Following the finding that hydrogen sulfide regulates KATP channel activity, subsequent studies have looked at the role of hydrogen sulfide on pancreatic islet cell insulin secretion which is reported to be regulated by KATP channels (89). Hydrogen sulfide has been reported to stimulate pancreatic islet KATP channels at a dose of 100 μM resulting in an inhibition of insulin secretion in pancreatic islet cell lines; INS-1E (90), HIT-T15 (91) and MIN-6 (92). The activation of KATP channels caused membrane hyperpolarisation as a result of K+ efflux thus preventing the opening of voltage dependent Ca2+ channels; this prevents the influx of Ca2+ and secretion of insulin (91). A later study by Tang et al had corroborated these findings by showing that the L-type voltage-dependent Ca2+ channel was closed in
primary isolated islet cells treated with NaHS and the converse effect was observed with cells treated with a CSE inhibitor, D,L-propargylglycine (PAG) (93). Increased hydrogen sulfide synthesising enzyme expression and activity have been reported in the pancreas and liver of streptozocin-induced diabetic rats (94) and the inhibition CSE with PAG was shown to increase serum insulin level as well as lower hyperglycemia in Zucker diabetic rats (95).

Another well studied aspect of hydrogen sulfide is its involvement in cytoprotection in response to oxidative stress. As a reducing agent, hydrogen sulfide has been shown to scavenge hypochlorous acid (96) and peroxynitrite (97) in vitro resulting in the protection of human neuroblastoma cell line, SHSY5Y against oxidative stress. Protection was observed with NaHS concentrations of 30 to 250 μM per 125 μM hypochlorous acid and 300 μM peroxynitrite used. However it has been noted that the concentration of free tissue sulfide is far lower than other classical reducing agents such as GSH (intracellular level of 0.5 – 10 mM (98)) and therefore direct interaction of hydrogen sulfide with oxidants probably could not account completely for its protective effect (99). An indirect reducing effect was reported whereby hydrogen sulfide has been shown to enhance cystine/cysteine transporters thus resulting in increased GSH synthesis and distribution into the mitochondria with antioxidative tissue protecting effects (100, 101). Another proposed mechanism for the anti-oxidative effect of hydrogen sulfide is through the disruption of the upstream activator of the mitochondrial redox signalling molecule, p66shc. This has been shown to occur via the s-sulfhydration of the cysteine-59 residue of p66shc thus interfering with the phosphorylation of the serine-36 residue and ultimately preventing its activation (102).

Hydrogen sulfide has also been shown to play a nociceptive role with majority of the reports pointing towards a pronociceptive effect. Exogenous hydrogen sulfide was found to elicit pronociceptive response in the paw (1 nmol per rat and mouse paw)(103-105), colon (5 nmol per mouse intracolonically)(106, 107) and pancreas (500 nmol per rat pancreas)(108, 109) of mice and rats following administration of NaHS. Inhibition of endogenous hydrogen sulfide synthesis with propargylglycine (PAG) also resulted in an attenuation of pronociceptive signalling associated with conditions such as pancreatitis (108), bladder cystitis (110), spinal injury (111) and endotoxaemia induced paw oedema (105). Hydrogen sulfide has been shown to mediate pronociception through sensitization of the Ca.3.2 T-type calcium channel (108, 110, 111) and the calcium channel transient
receptor potential ankyrin-1 (TRPA1) (105, 112, 113). Hydrogen sulfide has also been shown to activate the nociceptive transient receptor potential vanilloid-1 (TRPV1) in airway inflammation (114) and gut signalling (79) though not in the context of pain development.

Endogenous hydrogen sulfide synthesized via CSE regulates cellular apoptosis. This has been demonstrated primarily in pancreatic cells (17, 69, 115) and also observed in lung tissue (116). In pancreatic beta cells, endogenous hydrogen sulfide was found to be anti-apoptotic in response to glucotoxicity in vitro (17) and in vivo (117) as well as pro-apoptotic in streptozocin induced diabetes in rats (115). In pancreatic acinar cells, CSE inhibition increased apoptosis in response to pancreatitis (69). Exogenous hydrogen sulfide has also been shown to influence apoptosis. Direct treatment with hydrogen sulfide in the micromolar range was shown to be pro-apoptotic in pancreatic acinar cells (118), human aortic smooth muscle cells (119) and human pulp stromal cells (120) without any underlying pathological conditions. However, exogenous hydrogen sulfide has been found to play an anti-apoptotic role when administered during pathologies. It was found to be anti-apoptotic in cardiomyocytes in response to myocardial infarction (100 μM NaHS treated cardiomyocytes and 54 μmol NaHS/ kg treated rat) (121, 122), hepatocytes and tubular kidney cells in response to ischemia reperfusion injury (13 μmol Na₂S/ kg mouse and 100 μmol NaHS/ kg rat respectively) (123, 124) as well as lung tissue in response to allergy (116). Although the role of hydrogen sulfide in apoptosis is mixed, there appears to be a pattern distinguishing the effects of endogenous versus exogenous hydrogen sulfide and whether there is an underlying pathology.

Hydrogen sulfide has long been known to disrupt the mitochondrial respiratory chain by inhibiting cytochrome C oxidase (125). However low levels of exogenous hydrogen sulfide (6-8 nmol/min (126) and 0.1-1 μM (127)) have also been shown to increase mitochondrial ATP production in colonocyte (126) and liver cells (127) while excursion out of this narrow range (>6-8 nmol/min (126) and 3-30 μM (127)) were shown to inhibitory. Endogenous hydrogen sulfide has also been shown to produce a similar effect; activation of vascular smooth muscle cells (vSMCs) was reported to cause CSE translocation from the cytosol to the mitochondria which lead to increased mitochondrial hydrogen sulfide synthesis (from <0.1 to 0.5 nmol/g/min), hydrogen sulfide content (from 4 to 12 nmol/g) and ATP production, CSE gene deletion abolished these effects (128). Mitochondrial 3MST was also shown to contribute to ATP turnover via hydrogen sulfide synthesis (127). Supplementation of liver

16
mitochondrial lysates with 3-maercaptopyruvate (3-MP), a substrate of 3MST, resulted in a dose dependent increase in hydrogen sulfide synthesising activity up to 10 µmol/g/min with 30 µM 3-MP, and intact mitochondria exhibited dose dependent increased ATP turnover when treated with increasing concentrations of 3-MP (10 to 100 nM). These effects were abolished with 3-MST knockdown using siRNA (127). The hydrogen sulfide mediated contribution to mitochondrial ATP turnover has been shown to occur via reduction of sulfide quinone oxidoreductase (SQR) (127, 129). Hydrogen sulfide enters the mitochondrial respiratory chain by reducing SQR at its cysteine disulfide such that a persulfide group is formed at one of the cysteines (SQR-SSH) The electrons are fed into the respiratory chain via the quinone pool (Q<sub>ox</sub>/Q<sub>red</sub>), and finally transferred to oxygen by cytochrome oxidase (complex IV) (126, 129).

A striking observation of the studies mentioned here is the amount of exogenous hydrogen sulfide used to treat cells and animals appears to be multiple orders more than the amount of free tissue hydrogen sulfide reported (based on Levitt et al.’s measurements which is currently the most sensitive and specific method (37)). Assuming we used Levitt et al.’s reported liver levels of free hydrogen sulfide (Table 1) to extrapolate the total amount present in a mouse or rat, an average mouse (25 g) would have less than 2.5 nmol free hydrogen sulfide per mouse and an average rat (150 g) 15 nmol/ rat. These values are much lower than the reported low micromole and high nanomole levels of exogenous hydrogen sulfide injected into mice and rats and used to treat cells. However it would be interesting to note that a detailed study by Vitvitsky et al (33) on endogenous hydrogen sulfide turnover has shown a high rate of hydrogen sulfide synthesis in mouse liver supplemented with 0.1 mM cysteine; 484 umol/h/kg which could be extrapolated to 12.1 umol /h/mouse and 72.6 umol/h/rat for comparison. The authors also describe a high turnover rate of endogenous hydrogen sulfide due to its rapid catabolism and consumption thus suggesting an underestimate of the true rate of endogenous hydrogen sulfide synthesis. As a result of this high turnover, their calculated steady state of hydrogen sulfide level in the liver is 29 nmol/kg, (extrapolated to ~0.725 nmol/ mouse and 4.35 nmol/ rat – assuming weights described before) which once again is below the exogenous hydrogen sulfide doses used to treat these animals and in vitro cells. However, this reported high rate of synthesis and turnover suggests a constant state of endogenous hydrogen sulfide flux and the detectable levels of free tissue hydrogen sulfide at any point in time would not truly reflect the amount that is being...
generated. Therefore the question of whether the exogenous hydrogen sulfide doses used to elucidate its physiological effects is physiologically relevant is still an open one. Additionally, many of these studies also used endogenous hydrogen sulfide synthesising inhibition to corroborate their findings thus supporting the physiological relevance of these reported hydrogen sulfide mediated roles.

Figure 1.4 Possible physiological effects of hydrogen sulfide. Illustration of the current known physiological effects of hydrogen sulfide.

1.4 Hydrogen sulfide reactivity and mode of action

The major catabolic pathway for hydrogen sulfide is via a series of redox conversions in the mitochondria that ultimately leads to the formation of thiosulfate (130). This pathway is mediated by three enzymes, sulfidequinoneoxido-reductase (SQR) is first reduced by hydrogen sulfide at the
external disulphide to form a thiol (R-SH) and a persulfide (R-S-SH). This two electron oxidation of hydrogen sulfide reduces the FAD prosthetic group, which uses ubiquinone as an electron acceptor. The sulfur atom on the persulfide is a reactive sulfane (S\text{0}), which is oxidized by a sulfur dioxygenase enzyme encoded by the gene ET\text{HE1}, consuming O\text{2} and H\text{2}O to form sulfite (H\text{2}SO\text{3}). The final reaction is catalyzed by a sulfur transferase, rhodanase, which produces thiosulfate (H\text{2}S\text{2}O\text{3}) by transferring a second persulfide from the SQR to sulfite. This is illustrated as below in the mitochondria:

Apart from this catabolic pathway, hydrogen sulfide has recently been shown to physiologically react with other molecules owing to the nucleophilic capacity of the hydrosulfide ion (HS\text{-}) (131). The pK\text{1} of hydrogen sulfide is 6.76 at 37°C, therefore at pH7.4 about four fifths of hydrogen sulfide is present as the reactive HS\text{-} ion (28). Spiked exogenous hydrogen sulfide is quickly scavenged in biological solutions such as plasma (32), tissue homogenates (33) and even a pure protein solution of 5% BSA (32) with reported half-lives of 120 s in mouse liver homogenates (33) and 130s in rat plasma (32). Currently, there are three general forms of hydrogen sulfide interaction within the biological milieu reported under physiological conditions; as a reductant (132), through protein s-sulfhydration (31) and interaction with metal ion containing molecules (133). The discovery of these interactions have also led the elucidation of the direct targets and mechanisms by which hydrogen sulfide exerts its observed physiological effects. This section will describe the reported targets of hydrogen sulfide interactions and its subsequent physiological implications.
1.4.1 Reductant

Due to the nucleophilic nature of the predominant hydrogen sulfide (HS\textsuperscript{-}) species under physiological conditions, most of the hydrogen sulfide interactions in the biological milieu are a result of its reducing ability. However, in this section the capacity of hydrogen sulfide as a reductant will be described in the context of an antioxidant and its interactions with endogenous free radicals. Hydrogen sulfide has been shown to scavenge free radicals such as peroxynitrite (96) and hypochlorite (HOCl) (97) in cultured neuronal cells. An equimolar solution of GSH and hydrogen sulfide have been shown to be equally effective in preventing protein nitrotyrosination by peroxynitrite (96) and protein oxidation by hypochlorite (97) which resulted in a protection of cultured neuronal cells against oxidative stress. The proposed mechanism of hydrogen sulfide reaction with peroxynitrite by Carballal et al involves the nucleophilic attack of the anionic form of hydrogen sulfide (HS\textsuperscript{-}) on the peroxidic oxygen of peroxynitrous acid, leading to the formation of nitrite and dihydrogen disulfide presumably with the formation of sulfenic acid (HSOH) as intermediate:

\[
\begin{align*}
\text{HS}^- + \text{ONOOh} & \rightarrow \text{HSOH} + \text{NO}_2^- \\
\text{HS}^- + \text{HSOH} & \rightarrow \text{HSSH} + \text{OH}^- 
\end{align*}
\]

Nagy et al reported a mechanism of hydrogen sulfide reaction with hypochlorite analogous to that described for cysteine in which the initial oxidation step is followed by the formation of a dihydrogen disulfide (HSSH) (134):

\[
\begin{align*}
\text{HS}^- + \text{HOCl} & \rightarrow \text{HSCl} + \text{OH}^- \\
\text{HSCl} + \text{H}_2\text{O} & \rightarrow \text{HSOH} + \text{Cl}^- + \text{H}^+ \\
\text{HS}^- + \text{HSOH} & \rightarrow \text{HSSH} + \text{OH}^- 
\end{align*}
\]

However Carballal et al reported that the likelihood of hydrogen sulfide serving as a physiologically relevant antioxidant appears unlikely compared to other abundant intracellular thiols such as GSH and cysteine with similar rate constants to a variety of oxidants including peroxynitrite (hydrogen sulfide : 4.8×10\textsuperscript{3} M\textsuperscript{-1} s\textsuperscript{-1}, GSH : 1.3×10\textsuperscript{3} M\textsuperscript{-1} s\textsuperscript{-1}, cysteine : 4.5×10\textsuperscript{3} M\textsuperscript{-1} s\textsuperscript{-1}, pH 7.4, 37 °C) and hypochlorite (hydrogen sulfide : 8×10\textsuperscript{7} M\textsuperscript{-1} s\textsuperscript{-1}, pH 7.4, 37 °C and GSH : ≥10\textsuperscript{7} M\textsuperscript{-1} s\textsuperscript{-1}, cysteine : 3.2×10\textsuperscript{7} M\textsuperscript{-1} s\textsuperscript{-1}, pH 7.4, 25 °C)(99) due to the lower bioavailable levels of endogenous hydrogen sulfide.
These claims were countered by Fillipovic et al that reported a slightly higher hydrogen sulfide mediated peroxynitrite scavenging rate constant \((4.8 \times 10^3 \text{ M}^{-1} \text{s}^{-1}, \text{pH 7.4, 37 }^\circ\text{C})\) attributed to the difference in hydrogen sulfide source and method employed. They have reported the mechanism to be associative than multi-step free-radical as expected for other thiols without the need for oxygen consumption. This is in agreement with a primary formation of a new reaction product characterized by spectral and computational studies as thionitrate \((\text{HSNO}_2)\). The proposed reaction mechanisms are as follows:

\[
\begin{align*}
\text{HS}^- + \text{ONO}_2^- & \rightarrow \text{HS}^- + \cdot\text{NO}_2 + \text{OH}^- \\
\text{HS}^- + \cdot\text{NO}_2 & \rightarrow \text{HSNO}_2
\end{align*}
\]

Fillipovic et al further demonstrated the protective effect of hydrogen sulfide against an equimolar concentration of peroxynitrite in cultured HeLa and Jurkat cells against oxidative stress (135). The authors discussed that intracellular biochemical reactions are greatly affected by the constraints of high internal concentrations of macromolecules and cellular architecture thus creating a crowded or volume occupied space. In such a situation, the relative size, shape and diffusibility of a molecule could significantly alter its reactions rates (136). This speaks in favour of hydrogen sulfide from the more ubiquitous intracellular reductant GSH being a smaller and highly diffusible molecule without the need of specific transporters (135) (137).

1.4.2 Protein persulfide formation/ s-sulfhydration

The existence and biological significance of protein persulfides or sulfane sulfur containing proteins predates the earliest report of hydrogen sulfide mediated physiological relevance (138, 139). In 1970, Cavallini et al had shown the capacity for exogenous hydrogen sulfide (in the form of dissolved sodium sulfide) to cleave disulfide bonds of purified proteins under controlled experimental conditions to yield protein persulfides (138), however it was not certain then if this process occurred under physiological conditions and more so, if endogenously synthesised hydrogen sulfide could contribute to the generation of protein persulfides. Therefore in the advent of current hydrogen sulfide studies, the formation of protein persulfide has once again resurfaced and thought to be a possible mechanism by which hydrogen sulfide may exert its observed physiological effects (140).
In 2008, Mustafa et al had reported the ability of hydrogen sulfide to s-sulfhydrylate proteins to yield a protein persulfide using a modified biotin switch assay (31). They had subsequently estimated that 10 to 25% of numerous liver proteins are s-sulfhydrated under physiological conditions and that the resulting protein persulfides possess altered activity compared to thiol counterparts; for example s-sulfhydrated GAPDH and beta-actin showed a 30% enhanced activity and polymerization ability respectively compared to their thiol counterparts (31). Following this report, there have been an increasing number of studies that identified specific inducible s-sulfhydrated proteins under (patho)physiological conditions presumably through the action of hydrogen sulfide; they include the Kir 6.1 subunit of the K<sub>ATP</sub> channel (141), p65 subunit of NFκB (142), Protein tyrosine phosphatase 1B (143), Kelch-like ECH-associated protein-1 (144) (145), p66Shc (102) and mitogen-activated protein kinase kinase 1 (146). The s-sulfhydration of these proteins either led to its enhanced activity or inhibition and suggests a direct mechanism by which endogenous and exogenous hydrogen sulfide exerts its (patho)physiological effects; they include vasorelaxation via activation of the K<sub>ATP</sub> channels (141), cytoprotection via NF-E2 p45-related factor 2 (Nrf2) (144, 145) and Poly (ADP-ribose) polymerase activation (146), apoptotic control via NFκB activation (142) and anti-oxidative effect via inactivation of p66Shc in the mitochondria (102). The methods used to identify protein persulfides will be elaborated in section 2.1.2.

Despite the growing reports on the incidences of physiological hydrogen sulfide mediated protein persulfide formation, there has been little information on the nature of this reaction. A direct reaction between hydrogen sulfide (H₂S or HS⁻) and free thiols (R-SH) to form a persulfide is not possible as both sulfurs are in the same oxidation state of -2. Hydrogen sulfide has however been reported to generate protein persulfides when it reacts with oxidised free thiols and disulfides (147, 148). Therefore protein s-sulfhydration has been postulated to take place by two methods; (i) nucleophilic attack of sulfide on oxidised cysteine residues such as cysteine sulfenic acids (R-Cys-SOH) or cysteine disulfides (R-S-S-R)(147); (ii) reaction between cysteine thiols and oxidised sulfide species such as polysulfides (R-S-Sₙ-S-S-R) as the S° atom in polysulfides with its 6 valence electrons is known to enter and exit sulfur structures readily with the thiosulfoxide tautomer as an intermediate (134, 148). These reactions are illustrated in Figure 1.5.
Figure 1.5 Formation of protein persulfides (s-sulfhydrated proteins) by hydrogen sulfide. Protein persulfides can be formed by (i) nucleophilic attack of sulfide on oxidised cysteine residues such as cysteine sulfenic acids (a) or cysteine disulfides (b) and (ii) reaction between cysteine thiols and oxidised sulfide species such as polysulfides (c) as the S\(^{\text{I}}\) atom in polysulfides with its 6 valence electrons is known to enter and exit sulfur structures readily with the thiosulfoxide tautomer as an intermediate (148).

The biological relevance of a persulfide protein as opposed to its thiolated state has been the subject of continuous study. It has been shown that persulfides are better nucleophiles, reductants and acids compared to their thiol counterparts due to the α effect (149, 150) and this is thought to create a “hyperactivated” state of thiol containing proteins that possess this functional group (147). A direct example of this is the enhanced reducing ability of the classic antioxidant GSH; GS-SH was shown to be 20 and 5 fold more efficient at reducing ferric iron than GSH and hydrogen sulfide respectively (147). This is supported by a subsequent report that further showed high and maintained levels of endogenously produced GSH persulfide and other cysteine persulfides in the plasma, cells and tissues of humans and mammals (151).

The formation of protein persulfide through the reducing ability of hydrogen sulfide provide a possible direct link between hydrogen sulfide and its observed (patho)physiological effects. Apart from modifying protein interaction and activity, the sulfane sulfur group of the persulfides also serves as a source of hydrogen sulfide as mentioned in section 1.2.2. The amount of sulfane sulfur present in human circulation was estimated to be ~1 µM using DTT as the reducing agent to liberate sulfane sulfur.
followed by free hydrogen sulfide derivatisation to a fluorescent thionine end product and detection by HPLC (152).

1.4.3 Interaction with metal ions and metal containing proteins

Metal ions are ubiquitous in living organisms and are responsible for numerous biological roles such as charge conductance and signalling, mediating redox reactions, essential cofactors for enzymatic activity and as structural supports (153). Hydrogen sulfide is able to bind to metal ions particularly in its $\text{S}_2^-$ state in an aqueous solution to form metal sulfide precipitates (154). This has been employed by some microorganisms as a strategy to scavenge extracellular metal ions and therefore prevent heavy metal accumulation and toxicity (155, 156). However evidence of physiological hydrogen sulfide binding to free metal ions in mammals and humans and its biological significance is scarce. There has been one study that links hydrogen sulfide ability to activate T-type Ca$^{2+}$ channels via the chelation of luminal Zn$^{2+}$ ions in the colon of mice, thus explaining the pro-nociceptive effect of intracolononic administration of exogenous hydrogen sulfide (107).

The binding of hydrogen sulfide to metal ion containing proteins however is better established (157). The most well documented incidence in humans is the binding of hydrogen sulfide to haemoglobin to form sulfhaemoglobin due to occupational and environmental exposure thus impairing the transport of oxygen in the affected individual (158). Early studies have also shown hydrogen sulfide to interact with other heme containing proteins such as catalase (159) and lactoperoxidase (160) in vitro resulting in an inhibition of activity. More recent studies have shown hydrogen sulfide to interact and inhibit the myeloperoxidase (MPO) enzyme (161-163). This was proposed to occur via the interaction of the enzyme in its MPOFe(II) or MPOFe(III) state with hydrogen sulfide to form a ferrous-MPO-hydrogen sulfide complex which therefore acts as a sink because it traps MPO in an inactive form when sulfide is present (163). The reported IC$_{50}$ for this sulfide mediated inhibitory mechanism of MPO is 1 µM in the presence of 5-60 µM peroxide range (163) suggesting its biological relevance. Apart from iron containing proteins, hydrogen sulfide was also shown to inhibit the enzymatic activity of the angiotensin converting enzyme (ACE) in endothelial cells through the interaction of hydrogen sulfide with zinc(II) ion in the active centre of the protein (133).
Approximately a quarter to a third of all proteins require metals to carry out their functions (164). These metal ions may confer redox capabilities in the case of iron containing enzymes or structural/recognition motifs such as zinc fingers among many others (164). Therefore the ability for hydrogen sulfide to bind to metal ions within proteins provide an avenue for which it may modulate cellular activity through the plethora of metalloproteins along with its diverse functions.
**Table 2 Summary of hydrogen sulfide reactivity and mode of action.** Description of methods of hydrogen sulfide interaction within the biological milieu reported under physiological conditions; as a reductant (132), through protein s-sulfhydration (31) and interaction with metal ion containing molecules (133) Abbreviations; PTP1B - protein-tyrosine phosphatase 1B, Keap1 - Kelch-like ECH-associated protein 1, Nrf2 - NF-E2 p45-related factor 2, MEK1 - mitogen-activated protein kinase kinase 1, PARP-1 - poly(ADP-ribose)ation polymerase-1.
1.5 Hydrogen sulfide and inflammation

1.5.1 Inflammation

Inflammation is a complex set of interactions among soluble factors and cells that stem from an injury or insult. These interactions lead to the activation of the immune system to contain and neutralize the source of the insult followed by resolution and tissue repair. These complex interactions can be broken down to a generic pathway consisting of inducers, sensors, mediators and effectors (169). The progress along this pathway relies on a series of checks and balances to ensure an appropriate and regulated activation of the inflammatory response followed by subsequent resolution (170). The main inducer and sensor of inflammation involves the recognition of microbes by the innate immune system. This occurs via the recognition of highly conserved features on microbes (pathogen associated molecular patterns (PAMPs)) or specific endogenous molecules (damage associated molecular patterns (DAMPs)) by transmembrane and cytosolic pattern recognition receptors (PRR) such as Toll-like receptors (TLR), Nod-like receptors (NLR) and RIG-1 like receptors (RLR) in innate response cells (171). The bulk of early sensing by the innate system is performed by tissue resident macrophages, dendritic cells and mast cells (171). Activation of the PRRs on these cells initiates a signalling cascade that results in the synthesis and release of inflammatory mediators which can be grouped into seven categories; vasoactive amines (i.e. histamine and serotonin), vasoactive peptides (i.e. substance P and fibrin degradation products), fragments of complement components (C3a, C4a and C5a), lipid mediators (eicosanoids and platelet-activating factors), cytokines (TNF-α, IL-1, IL-6 etc.), chemokines (MCP-1, MIP-2α etc.) and proteolytic enzymes (i.e. elastin, cathepsins and matrix metalloproteinases) (169). The release of these mediators then activates the effectors comprising of tissues and cells resulting in the recruitment of activated immune response cells to the site of injury/infection through extravasation (172) to contain and neutralize the insult.

Typically, neutrophils are the first of the immune cells to be recruited to the site of inflammation followed by monocytes (171). Neutrophils possess an arsenal of potent effector mechanisms to achieve its microbicidal role including phagocytosis as well as production of reactive oxygen species (i.e. $O_2^-$, $H_2O_2$ and HOCl), proteinases (ie serine-proteases, metalloproteases, thiol-proteases, and aspartate proteases) and bactericidal proteins (i.e. bacteriacidal/permeability increasing protein,
defensins and lactoferrin) (173). Together, these effector molecules are able to degrade proteins, disrupt lipids and damage DNA. Recruited and activated neutrophils then undergo a process that mobilizes its defense machinery that involves full assembly of NADPH oxidase at its plasma membrane thus generating the reactive oxygen species that would in turn serve as a substrate for MPO enzyme to generate HOCl, a potent oxidant, as well as express a slew of pro-inflammatory cytokines and chemokines that further propagate the inflammatory response (173). A prolonged activation of neutrophils and mast cells would in turn condition the activation of professional antigen presenting cells, most notably the dendritic cells (DCs) (170). These activated DCs would then pick up antigens from the site of insult and travel to the lymph nodes where they would activate the naïve T and B lymphocytes thus initiating the adaptive immune response (174).

Successful elimination of the source of insult should progressively lead to the termination of the inflammatory response and ultimately resolution. This process involves the progressive decrease in neutrophil recruitment (via changes in cytokine and chemokine profile to a more anti-inflammatory or resolution state) and neutrophil apoptosis and clearance by macrophages that in turn inhibit the pro-inflammatory cytokine, chemokine and eicosanoid production of the ingesting macrophages (173). Despite the numerous points of control, the immune response is not a faultless system, aberrant and hyperactivation of the immune response can occur leading to immunopathological events at the detriment of the host (175). Aberrant and chronic activation of the immune response leads to autoinflammatory and autoimmune diseases; both are initiated by the innate immune response but the latter involves the adaptive immune response as well (176). This occurs when the immune system recognizes and reacts with endogenously synthesized molecules resulting in a loss of tolerance. Pattern recognition receptors (PRRs) and in particular TLRs play a crucial role in the early detection of PAMPs as well as DAMPs resulting in the subsequent activation of the innate and adaptive immune response.

It is thought that this pathway in immune activation results in the onset of autoinflammatory and autoimmune diseases initiated through apoptosis and subsequent presentation of autoantigens from the dying cells to antigen presenting cells (177). The activity of nuclease and proteases during the apoptotic process could potentially generate neo-epitopes that can be recognized as foreign. Neo-epitopes could also be formed by other forms of post-translational modification or through structural modifications brought about by environmental factors. These autoantigens also serve as
autoadjuvants as they are recognized by the innate system therefore activating the whole cascade of immune response (177). Autoimmune and autoinflammatory diseases are associated with chronic pathologies such as Crohn’s disease, PAPA syndrome, systemic lupus erythematosus and rheumatoid arthritis (176). While these conditions are debilitating, they rarely lead to direct mortality. However, chronic inflammation brought about in such pathologies are strongly linked to the development of cancer (2).

Another type of immunopathology involves the hyper-responsiveness of the immune system towards pathogenic or non-pathogenic stimuli. Localized inflammation is a physiological protective event targeted at controlling and eliminating the source of an insult, however loss of this control results in an overly activated immune response and could lead to the development of a systemic response known as systemic inflammatory response syndrome (SIRS) (178). SIRS is associated with a massive increase in plasma pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 thus initiating inflammatory events distant to the source of the insult. If left unresolved, SIRS could progress to multiple organ damage syndrome (MODS) and failure to maintain homeostasis without intervention resulting in high mortality rate of between 40% - 98% depending on the number of organs affected (179). SIRS and MODS are common complications in pathogenic derived pathology such as sepsis (30%) and non-pathogenic derived pathology such as acute pancreatitis (24%), burns (40%) and trauma (30%) (178).

During the progression of SIRS, the body launches an anti-inflammatory effort in order to regulate the immune response. This involves a systemic release of anti-inflammatory molecules such as interleukins-4, -10, -11, and -13; transforming growth factor-beta; colony-stimulating factors; soluble receptors to tumour necrosis factor; and receptor antagonists to IL-1 (180). The result is immune suppression, which can sometimes be profound. If the compensatory anti-inflammatory reaction is sufficiently severe, it will manifest clinically as anergy, an increased susceptibility to infection or both. This response is termed compensatory anti-inflammatory response syndrome (CARS) (180). Therefore there exists a continuum in systemic immunopathologies between SIRS and CARS where one may predominate over the other with mid-points where both responses exist and is termed mixed anti-inflammatory response syndrome (MARS).

The activation of the inflammatory response though essential is generally associated with collateral damage to surrounding healthy host tissue as the arsenal employed by the immune response cells are
not able to distinguish between self and non-self (ie reactive oxygen species and proteases), particularly with respect to the innate immune response cells and neutrophils in particular. This activation is usually regulated and kept under control though over-compensation leading to suppression of the immune response may arise. Therefore dysregulation of the inflammatory response leads to disorders and disease (170) and a better understanding of the machinery and signalling involved in the inflammatory response could lead to potential therapeutic targets in immunopathological conditions. However such therapeutics should take into account the inflammatory state of the disease as the choice of immunomodulation therapies would depend on whether SIRS or CARS predominate upon presentation.

**Figure 1.6 Illustration of the innate inflammatory response.** Pathogenic insult initiates an inflammatory response by either releasing pattern associated molecular pattern molecules (PAMPs) (ie lipopolysaccharide) or causing damage and lysis in host cells resulting in release of damage associated molecular pattern molecules (DAMPs). These molecules are sensed by the surrounding cells and resident macrophages resulting in macrophage activation. Activated macrophage releases a slew of pro-inflammatory mediators consisting of cytokines, chemokines and eicosanoids. These mediators cause physiological changes in vascular endothelial cells such as expression of adhesion molecules and leakiness of the vasculature resulting in recruitment and extravasation of circulating neutrophils and monocytes. These cells then migrate to the site of injury/insult following the chemokine/ eicosanoid gradient to neutralise and eradicate the source of the injury/insult. This could involve the generation of
ROS, digestive enzymes, metal ion chelators and phagocytosis. Recruited inflammatory cells also generate pro-inflammatory cells in response to inflammatory cues at the site of injury thus propagating the inflammatory response. Once the source of injury/insult is neutralised, the inflammatory response switches to a resolution phase, failure to do so results in an uncontrolled inflammatory response.

1.5.2 Hydrogen sulfide physiology and inflammation

Hydrogen sulfide has been shown to be involved in various physiological aspects related to the inflammatory response. As mentioned in section 1.3, endogenous hydrogen sulfide is involved in maintaining vascular tone by acting as a vasodilator. The vasodilatory effect is shown to be primarily mediated through the relaxation of smooth muscle cells via activation of the $K_{ATP}$ channels (141). Hydrogen sulfide could also influence vasotone via reduction of circulating angiotensin II, a known vasoconstrictor (181), by inhibiting ACE activity (133). Elevated levels of the renin-angiotensin system (RAS) activity have been demonstrated in diseases such as hypertension, cardiovascular diseases (myocardial infarction and atherosclerosis), kidney diseases and diabetes (182). Alterations in vascular tone affects blood flow and the inflammatory response. For example, vasodilation leads to increased blood flow and in an inflammatory response, this would increase the recruitment of leukocytes to the site of injury (183). Also, severe fall in blood pressure (hypotension) leading to dysfunction or failure of major organs is associated with systemic inflammatory conditions such as sepsis and endotoxemia. An increase in endogenous hydrogen sulfide has been associated with the development of sepsis, endotoxin and hemorrhagic induced shock (184, 185) though recent views suggest a more cautious interpretation largely due to the difficulty and variable methods employed in estimating endogenous levels of free hydrogen sulfide (186). In addition, perturbation of endogenous hydrogen sulfide levels by inhibition with PAG or use of a hydrogen sulfide donor such a NaHS was found to both increase mean arterial pressure in hemorrhagic induced shock (184) as well as decrease mean arterial pressure in septic mice (187) respectively. In the latter, it was further shown that the increased microvascular permeability by treatment with NaHS improved leukocyte recruitment to the site of infection thus improving the survival outcome in severe sepsis (187) which was in contrary to another report that corroborated hydrogen sulfide trafficking of leukocytes (188) but this was shown to have deleterious effects (189). Therefore the vasomodulatory effect of hydrogen sulfide appears to have a role in the inflammatory response.
In an inflammatory response, there is an accumulation of cytotoxic substances produced by the immune cells to neutralize and eliminate the source of the insult. These cytotoxic substances such as reactive oxygen/nitrogen species and pro-inflammatory cytokines do not distinguish between host and pathogen resulting in damage of the surrounding healthy host cells. This collateral damage is particularly devastating in autoimmune and autoinflammatory diseases where inflammatory response persists without the presence of a pathogen or the inability to eradicate a pathogen. Hydrogen sulfide has been shown to exert cytoprotective effects largely via its antioxidant and anti-apoptotic roles (190, 191), as mentioned in section 1.1.2 and 1.3.1. Administration of hydrogen sulfide to autoinflammatory pathologies such as myocardial infarction (121, 122) and ischemia reperfusion (123, 124, 192) was found to lessen the severity of healthy tissue destruction owing to its cytoprotective properties.

Nociception occurs via sensory neurons comprising of small diameter C-fibre and A\(\delta\) fibres (193). Excitation of these neurons via harmful stimuli such as thermal extremes, noxious chemicals and excessive pressure elicits a pain response as a method to sense harm. However, the excitation of these neurons have also been associated with the elicitation of an inflammatory response; stimulated sensory neurons release pro-inflammatory neuropeptides such as substance P (SP) and calcitonin gene-related peptide (CGRP) that interact with endothelial cells, mast cells, immune cells, and arterioles to initiate an inflammatory response termed neurogenic inflammation (194). As mentioned in section 1.1.2 hydrogen sulfide has been shown to sensitize nociceptors such as T-type calcium channel (108, 110, 111), TRPA1 (105, 112, 113) and TRPV1 (79, 114). Among these three receptors, TRPV1 and TRPA1 activation and association with neurogenic inflammation have been well established (195, 196). Functional TRPV1 and TRPA1 receptors are also present on non-neuronal cells such as keratinocytes and immune cells and similar to sensory neuronal cells, the activation of these receptors also influence the inflammatory response of these non-neuronal cells (197). Therefore the ability of hydrogen sulfide to sensitize nociceptors suggests a possible role of hydrogen sulfide in promoting neurogenic inflammation. This has been demonstrated in studies of airway inflammation (114) and sepsis (198) via activation of TRPV1.
Figure 1.7 The effect of hydrogen sulfide on the inflammatory response based on its reported physiological roles. As a vasodilator, hydrogen sulfide could lead to increased blood flow and vascular permeability leading to increased recruitment of leukocytes, vasodilation could also contribute to hypotension and shock. Hydrogen sulfide is also reported to be cytoprotective mainly due to its antioxidant (direct and indirect) and apoptotic effects. This could lessen the amount of collateral damage to the surrounding healthy cells due to ROS generated by neutrophils and macrophages, additionally cellular death by apoptosis is more favourable over necrosis as it does not lead to uncontrolled release of DAMPs. As a nociceptor, hydrogen sulfide has the potential to elicit a neurogenic inflammatory response by stimulating the release of pro-inflammatory neuropeptides such as substance P (SP) and calcitonin gene-related peptide (CGRP) that interact with endothelial cells, mast cells, immune cells, and arterioles to initiate an inflammatory response.

1.5.3 Direct effects of hydrogen sulfide on the inflammatory response

Apart from physiological events that influence the inflammatory response, hydrogen sulfide has also been shown to directly modulate the inflammatory response by activating molecules of the inflammatory cascade or reacting with the effector molecules generated by the immune response cells.

NFκB is a family of transcription factors that plays an important role in inflammation by promoting the transcription of pro-inflammatory genes (199). Endogenously synthesized as well as exogenously administered hydrogen sulfide has been associated with both activation and inhibition of NFκB in
response to inflammation. Endogenous hydrogen sulfide has been shown to activate liver and lung NF\(\kappa\)B following sepsis (200) and pancreatic NF\(\kappa\)B following pancreatitis (69). On the contrary, endogenous hydrogen sulfide is also associated with inhibition of NF\(\kappa\)B in the kidney following renal ischemia/reperfusion injury (124) and in macrophages upon LPS activation (201). Exogenously administered hydrogen sulfide increased NF\(\kappa\)B activation in primed monocytes (202), liver and lung following sepsis (200), LPS activated macrophages (203) and in the pancreas following pancreatitis (69). Like endogenous hydrogen sulfide, exogenous hydrogen sulfide has also been reported to inhibit NF\(\kappa\)B activation in the heart (122), retinal (204) and renal tissue (124) following ischemia/reperfusion injury as well as in LPS treated macrophages (201, 203). In the reports cited above, the hydrogen sulfide mediated modulation of NF\(\kappa\)B had been attributed to activation or inhibition of upstream signalling events. However, more recent reports have shown that hydrogen sulfide is able to directly s-sulfhydrate the p65 subunit of NF\(\kappa\)B on the cysteine-38 residue. The initial report of this finding showed that s-sulfhydration of the p65 subunit activates NF\(\kappa\)B (142), however a subsequent report that corroborates hydrogen sulfide mediated p65 subunit sulfhydration showed an opposite inhibitory effect (205). This was thought to be due to the different conditions under which the observation was made; physiological versus pathophysiological (205). Taken together, these findings propose a mechanism by which hydrogen sulfide may directly influence the progression of an inflammatory response through the modulation of NF\(\kappa\)B activity.

Nrf2 is another transcription factor that has been shown to be activated by hydrogen sulfide. Nrf2 is the primary transcription factor that binds to the antioxidant response element (ARE) in the regulatory regions of multiple genes and plays a key role in the suppression of the inflammatory response via regulation of the cytoprotective and antioxidant defense system (206). Nrf2 activity is negatively regulated by Keap-1 that functions an adaptor protein for a Cul3-based ubiquitin E3 ligase and binds to Nrf2 under normal conditions thus promoting its degradation by the proteasome (207). Hydrogen sulfide has been shown to sulfhydrate Keap-1 at cysteine 226 and 613 as well as cysteine 151 thus promoting its inactivation (144) and disassociation (145) from Nrf2 leading to activation of Nrf2. Exogenously administered hydrogen sulfide has been shown to exert an anti-inflammatory response through activation of Nrf2 in myocardial ischemia (208) (41), sepsis (209), diabetic nephropathy (210) and hemorrhagic shock (211).
Reactive oxygen and nitrogen species such as superoxide, nitric oxide, hydrogen peroxide, peroxynitrite and hypochlorous acid are among the effector molecules generated by immune response cells in an attempt to eradicate invading pathogens (212). Although effective, these potent free radicals are also capable of causing damage to surrounding healthy tissue through oxidative stress. As mentioned in section 1.4.1, hydrogen sulfide has been shown to be a strong antioxidant capable of scavenging peroxynitrite and hypochlorous acid with similar or greater reducing potential to ubiquitous endogenous antioxidants such as GSH and cysteine (99, 135). Administration of exogenous hydrogen sulfide has been shown to lessen the effect of oxidative stress in neuronal cells exposed to peroxynitrite (96), hypochlorous acid (97), glutamate (101) and beta amyloid (213). However, it is important to note that endogenous levels of hydrogen sulfide are substantially lower than the more common antioxidant, GSH, and it has been hypothesized that the possible direct contribution of hydrogen sulfide as a physiological antioxidant would be negligible. In fact most of the observed antioxidative protection afforded by hydrogen sulfide has been attributed to its ability to increase intracellular antioxidants such as GSH (214) (101). Despite the unfavourable endogenous levels, the relevance of hydrogen sulfide as a physiologically direct antioxidant has been argued owing to its small and diffusible nature making it more favourable in the crowded and confined environment of a cell compared to larger molecules such as GSH (215).

Hydrogen sulfide has been shown to modulate the activity of metal ion containing proteins by directly interacting with the metal ion (refer to section 1.3.3). It has been shown to inhibit MPO activity in primary human polymorphonuclear leukocytes (161) and in its purified form (162, 163) with an IC50 of ~1 μM (163). MPO is a crucial protein in the arsenal of innate immune response cells; it is responsible for the generation of large quantities reactive oxygen species with the purpose of killing pathogens. Hydrogen sulfide has also been shown to inhibit angiotensin-converting enzyme (ACE) activity in endothelial cells (133); the product of ACE is angiotensin II, the effector molecule of the renin-angiotensin system (RAS). RAS has been classically known for its role in maintaining blood pressure and water balance, however recent evidence have shown it to be involved in key events of the inflammatory process. Angiotensin increases vascular permeability via release of prostaglandins, regulates expression of adhesion molecules and chemokines by resident cells as well as directly activate infiltrating immunocompetent cells (182).
Figure 1.8 Illustration of direct reported effects of hydrogen sulfide on the inflammatory response.

Hydrogen sulfide affects the inflammatory response through its capacity as a reductant and ability to s-sulfhydrate as well as interact with metal ion containing proteins. Hydrogen sulfide has been shown to directly reduce reactive oxygen species such as peroxynitrite and hypochlorous acid, it is also capable of reducing extracellular cystine to cysteine thus facilitating transport via cysteine transporters (ASC) where it can serve as a substrate for GSH synthesis. Hydrogen sulfide is also known to s-sulfhydrate and regulate several key proteins related to the inflammatory response; activation of NFκB – transcription factor of numerous pro-inflammatory proteins, activation of Nrf2 – transcription factor of numerous anti-oxidative related proteins, p66shc – initiator of mitochondria mediated generation of ROS. Hydrogen sulfide is also reported to interact and inhibit the activity of key metal ion containing proteins; myeloperoxidase – responsible for the synthesis of inflammatory cell ROS, angiotensin converting enzyme – converts angiotensin I to angiotensin II which is reported to contribute to the inflammatory response via pro-inflammatory mediators such as cytokines, chemokines, eicosanoids and cell adhesion molecules.
1.5.4 Pro- or anti-inflammatory?

As described in section 1.5.2 and 1.5.3, it appears that hydrogen sulfide has the ability to function as both a pro and anti-inflammatory mediator via direct and indirect means. Indeed, the literature has equally mixed findings showing both pro and anti-inflammatory roles of hydrogen sulfide in multiple models of inflammatory diseases in animals. Animal studies aimed at elucidating the role of endogenous hydrogen sulfide through inhibition of the hydrogen sulfide producing enzyme, CSE, have shown both pro (44, 66, 189, 216-219) and anti (187, 220-224) inflammatory effects. Similarly, administration of exogenous hydrogen sulfide has also showed similar conflicting outcomes of pro (44, 45, 69, 189, 216, 218) and anti (187, 221, 223-229) inflammatory effects. These contrasting findings are further exemplified in similar models of inflammation such as sepsis (187, 189, 198, 209), pancreatitis (66, 69, 230), LPS induced endotoxemia (44, 231) and burn injury (45, 229) where both pro and anti-inflammatory effects of hydrogen sulfide have been reported.

Although the common aim of these studies is to determine the role hydrogen sulfide in the inflammatory response, there are differences between them that could result in the conflicting outcome. Firstly there is the issue of different animal models being used thus resulting with differing pathogenesis and subsequent elicitation of the inflammatory response. Hydrogen sulfide has been shown to be highly reactive with diverse physiological and pathophysiological roles. Therefore the interaction between hydrogen sulfide and different animal models may yield different outcomes that may be related to the pathogenesis of the disease of study.

Studies that administer exogenous hydrogen sulfide pose a much larger complication. To begin with, there is still very little agreement as to the amount of hydrogen sulfide present in circulation and tissues due to the difficulty in measuring free hydrogen sulfide (refer to section 1.2.3). Without accurately knowing physiological or pathophysiological levels of circulating and tissue free sulfide, it would be difficult to determine the amount of hydrogen sulfide to administer that would be (patho)physiologically relevant. This could create artifactual findings independent of the true role of endogenous hydrogen sulfide in the inflammatory response. Additionally, in studies with similar models of inflammation, there are inconsistencies with the dosage, source of hydrogen sulfide and route of administration. For example in the cecal ligation and puncture model of sepsis in mice, there are three research groups that utilise different dosage and route of administration. The groups that
administered NaHS at 100 µmol/kg (187, 209) reported an anti-inflammatory effect while 180 µmol/kg (189) was found to be opposite. In addition, one group used Lawesson’s reagent (that slowly releases hydrogen sulfide (232)) as one of their hydrogen sulfide source and there were differences in the timing of the doses (187). Another example is the burns injury model with one group administering 180 µmol/kg intraperitoneally (45) and another at 36 µmol/kg subcutaneously (229) with reports of pro- and anti-inflammatory effects respectively. These differences make it difficult to directly compare different studies and suggest caution when interpreting the role of hydrogen sulfide in inflammation by use of exogenous hydrogen sulfide donors.

Apart from simple salts such as NaHS and Na₂S that result in a large instant bolus release of hydrogen sulfide, there are also slow releasing donors that provide a sustained low level release of hydrogen sulfide, they include GYY4137 and S-diclofenac, a hydrogen sulfide-releasing dithiol-thione moiety attached by an ester linkage to diclofenac. These sustained slow releasing hydrogen sulfide donors have been shown to exert an anti-inflammatory effect in models of inflammatory diseases where NaHS was previously shown to be pro-inflammatory; this has been shown in pancreatitis (69, 230), LPS induced endotoxemia (44, 231) as well as LPS stimulated macrophages (203). Slow releasing hydrogen sulfide donors have also been shown to have anti-inflammatory effects in animal models of carrageenan induced hind-paw oedema (233), myocardial ischemia reperfusion injury (234) and acute knee joint inflammation (235). Interestingly, there have been no reports to date showing slow releasing hydrogen sulfide donors to be pro-inflammatory. This clearly suggests that the inflammatory effects of hydrogen sulfide are strongly dependent on the dose and rate of release/synthesis. This could perhaps relate to the affinity of hydrogen sulfide to its target molecules; at low concentrations of hydrogen sulfide it would most likely interact with the most abundant molecules present based on affinity, while at high concentrations of hydrogen sulfide it would be able to interact with less abundant molecules too. In an inflammatory response, there are large amount of reactive oxygen species generated as well as oxidised GSH (GS-SG) which could be the preferred target of hydrogen sulfide thus conferring an anti-inflammatory role. At higher concentration of hydrogen sulfide, it could interact with receptors and signalling molecules that are relatively less abundant thus initiating pro-inflammatory signalling response, for example NFκB (236).
Most studies looking at endogenous sulfide synthesis in inflammatory models use PAG as a pharmacological inhibitor to inhibit CSE activity; CSE is thought to be main contributor of hydrogen sulfide synthesis in the peripheral tissues while CBS in the brain (237). PAG is an irreversible inhibitor that targets pyridoxal-5-phosphate dependent enzymes, this includes the racemization, decarboxylation, transamination and β & γ elimination class of enzymes (238). Apart from CSE, PAG has also been shown to selectively inhibit methionine-γ-lyase (239), D,L amino acid oxidases (240), alanine transaminase (241) and aspartate aminotransferase (242). In a review by Whiteman et al (243), several non-CSE related effects of PAG that could directly or indirectly affect the inflammatory response have been discussed. It had also been noted that PAG rapidly accumulates in the circulation and D-PAG is metabolized to a yet unknown metabolite leading to renal injury. Therefore the non-CSE mediated actions of PAG coupled with the variety of pathologies as well as dosage regimes could potentially result with observable effects independent of endogenous hydrogen sulfide generation and explain the conflicting observation of both pro and anti-inflammatory effects of endogenous hydrogen sulfide.

Despite the limitations and contradicting results described above, the growing body of evidence suggests that CSE does play a role in the inflammatory response and this could be mediated through endogenous hydrogen sulfide synthesis. The current understanding of hydrogen sulfide role in inflammation could be further developed and made more accurate through the development and use of more sensitive and specific methods of detecting free hydrogen sulfide in biological solution and selective inhibitors towards endogenous hydrogen sulfide synthesizing enzymes.
<table>
<thead>
<tr>
<th>Model of disease/injury</th>
<th>Specie</th>
<th>Treatment</th>
<th>Parameter</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pro-inflammatory</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cecal ligation and puncture induced sepsis</td>
<td>Ms</td>
<td>PAG (50mg/kg) 1h pre or post</td>
<td>NaHS (180umol/kg) simul</td>
<td>Leukocyte infiltration, cytokines, NFκB</td>
</tr>
<tr>
<td>LPS induced endotoxemia 10mg/kg ip</td>
<td>Ms</td>
<td>PAG (50mg/kg) 30min pre</td>
<td>NaHS (30umol/kg) 30min pre</td>
<td>Leukocyte infiltration</td>
</tr>
<tr>
<td>LPS induced endotoxemia ~4ug/kg ip</td>
<td>Ms</td>
<td>PAG (113mg/kg) 30min pre</td>
<td>NaHS (180umol/kg) simul</td>
<td>Leukocyte infiltration, adhesion molecules</td>
</tr>
<tr>
<td>LPS induced endotoxemia 1mg/kg ip</td>
<td>Rt</td>
<td>PAG (50mg/kg) 5.5h post</td>
<td>NaHS (180umol/kg) simul</td>
<td>Leukocyte infiltration</td>
</tr>
<tr>
<td>Burns injury (30% exposure for 8s)</td>
<td>Ms</td>
<td>PAG (50mg/kg) 1hr pre or post</td>
<td>NaHS (180umol/kg) simul</td>
<td>Leukocyte infiltration</td>
</tr>
<tr>
<td>Caerulein induced Pancreatitis</td>
<td>Ms</td>
<td>PAG (100mg/kg) 1hr pre or post</td>
<td>NaHS (28umol/kg) 1h post</td>
<td>Leukocyte infiltration, cytokine, oxidative stress</td>
</tr>
<tr>
<td>Sodium Taurocholate induced pancreatitis</td>
<td>Rt</td>
<td>PAG (80mg/kg) 1h post</td>
<td>NaHS (50umol/kg) OD for 10d</td>
<td>Leukocyte infiltration, cytokine</td>
</tr>
<tr>
<td>Cisplatin induced renal damage</td>
<td>Rt</td>
<td>PAG (5mg/kg) BD for 4d</td>
<td>NaHS (100umol/kg) 1h pre</td>
<td>Leukocyte infiltration, cytokines</td>
</tr>
<tr>
<td>Gentamicin induced renal damage</td>
<td>Rt</td>
<td>PAG (25mg/kg) OD for 10d</td>
<td>NaHS (50umol/kg) OD for 10d</td>
<td>Cytokine, oxidative stress</td>
</tr>
<tr>
<td>Haemorrhagic Shock</td>
<td>Rt</td>
<td>PAG (50mg/kg) 30min pre bleed</td>
<td>NaHS (180umol/kg) simul</td>
<td>Leukocyte infiltration, cytokines</td>
</tr>
<tr>
<td>Carrageenan induced hind paw edema</td>
<td>Rt</td>
<td>PAG (50mg/kg) 1h pre</td>
<td>NaHS (36umol/kg)</td>
<td>Leukocyte infiltration, cytokines</td>
</tr>
<tr>
<td><strong>Anti-inflammatory</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cecal ligation and puncture induced sepsis</td>
<td>Ms</td>
<td>PAG (50mg/kg) 1h pre</td>
<td>NaHS/ Laws (10-100umol/kg) 1h pre</td>
<td>Leukocyte infiltration, adhesion molecules</td>
</tr>
<tr>
<td>LPS induced endotoxemia 10mg/kg ip</td>
<td>Ms</td>
<td>PAG (50mg/kg) 1h pre, 2h post, OD for 2d</td>
<td>S-Diclofenac (47.2umol/kg) 1h pre</td>
<td>Leukocyte infiltration, cytokines</td>
</tr>
<tr>
<td>LPS induced endotoxemia 4mg/kg iv</td>
<td>Rt</td>
<td>GYY4137 (50 mg/kg) 10min post</td>
<td>GYY4137 (50 mg/kg) 10min post</td>
<td>Cytokines, eicosanoid, nitric oxide, NFκB</td>
</tr>
<tr>
<td>Burns injury (40% exposure for 10s)</td>
<td>Ms</td>
<td>NaHS (36umol/kg)</td>
<td>NaHS (36umol/kg)</td>
<td>Leukocyte infiltration, cytokines</td>
</tr>
</tbody>
</table>
### Table 3 Summary of studies investigating the role of hydrogen sulfide in animal models of disease/injury.

This table shows reported role of hydrogen sulfide in the inflammatory response of various models of disease/injury in animals. These studies have either used the hydrogen sulfide synthesizing enzyme inhibitor, PAG or hydrogen sulfide donors as a means of modulating endogenous hydrogen sulfide levels. There are mixed results showing both pro and anti-inflammatory effects of hydrogen sulfide even with similar models of disease/injury. However there appears to be differences between studies such as variation in the choice of hydrogen sulfide donors, route of administration and dosage regime as well as method of inducing disease/injury. (Ms – mouse, Rt – rat, Pg – pig, OD – once daily, BD – twice daily, ip – intraperitoneal, sc – subcutaneous, iv – intravenous, ia – intraarticular, ic – intracolic)

<table>
<thead>
<tr>
<th>Disease Model</th>
<th>Species</th>
<th>Treatment Details</th>
<th>Route</th>
<th>Outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caerulein induced Pancreatitis</td>
<td>Ms</td>
<td>ACS15 (15mg/kg) 1h pre/post</td>
<td>ip</td>
<td>Leukocyte infiltration</td>
</tr>
<tr>
<td>Renal ischaemia reperfusion, 45 min</td>
<td>Rt</td>
<td>PAG (50mg/kg) 1h pre</td>
<td>ip</td>
<td>Leukocyte infiltration, NFkB, adhesion molecule, eicosanoid, nitric oxide</td>
</tr>
<tr>
<td>Renal ischaemia reperfusion, 60 min</td>
<td>Rt</td>
<td>NaHS (0.15umol) post</td>
<td>ip</td>
<td>Leukocyte infiltration</td>
</tr>
<tr>
<td>Renal ischaemia reperfusion, 60 min</td>
<td>Pg</td>
<td>Na,S (1.28umol/10min) pre/post (12.8umol/30min) post</td>
<td>iv</td>
<td>Leukocyte infiltration, cytokine</td>
</tr>
<tr>
<td>Haemorrhagic shock</td>
<td>Rt</td>
<td>PAG (50mg/kg) 10min pre reperfusion</td>
<td>iv</td>
<td>Nitric oxide, cytokines</td>
</tr>
<tr>
<td>Kaolin/Carrageenan induced knee joint arthritis</td>
<td>Rt</td>
<td>Na,S (5nmol/joint) 24h post</td>
<td>ia</td>
<td>Leukocyte infiltration</td>
</tr>
<tr>
<td>Carrageenan induced knee joint synovitis</td>
<td>Rt</td>
<td>PAG (0.47ug/joint) 1h pre</td>
<td>ia</td>
<td>Leukocyte infiltration, nitric oxide</td>
</tr>
<tr>
<td>Carrageenan induced hind paw edema</td>
<td>Rt</td>
<td>S-Diclofenac (11.8-47.2umol/kg) 3h pre</td>
<td>ip</td>
<td>Leukocyte infiltration, oedema, nitric oxide</td>
</tr>
<tr>
<td>Colitis</td>
<td>Rt</td>
<td>PAG (50mg/kg) BD for 7d</td>
<td>ip</td>
<td>Leukocyte infiltration, cytokine, eicosanoid, nitric oxide</td>
</tr>
<tr>
<td>Myocardial ischaemia reperfusion injury</td>
<td>Rt</td>
<td>NaHS (54umol/kg) 15min pre</td>
<td>iv</td>
<td>Leukocyte infiltration, NFkB, oxidative stress, adhesion molecule</td>
</tr>
</tbody>
</table>

#### 1.5.5 Therapeutic potential of hydrogen sulfide?

In order to consider hydrogen sulfide modulation as form of therapy, our current understanding of it should afford us satisfactory knowledge of the following; (1) predictable effect or outcome, (2) identification of an established and relatively specific target, and (3) useable/suitable therapeutic
window of application. Unfortunately with the current knowledge of hydrogen sulfide, we are still unable to definitively answer or demonstrate these points, particularly with respect to the inflammatory response.

As demonstrated in section 1.5.4, there are still very conflicting effects of hydrogen sulfide (by means of exogenous introduction or endogenous inhibition) on the inflammatory response, granted this could be due to several factors such as different disease models and methods of hydrogen sulfide modulation. However, this only contributes to the second point of target specificity. Hydrogen sulfide is a highly nucleophilic and diffusible gaseous molecule therefore making it highly motile and reactive. There is currently no known specific ‘receptor’ for hydrogen sulfide which brings into question what would the hydrogen sulfide-inflammatory studies above be targeting to bring about the effects? There are reports of its ability to react with thiol groups of numerous proteins to form protein persulfides thus increasing or decreasing its activity (refer to section 1.4.2 for details). This is by far the only well studied aspect and could speak of the various physiological effects ascribed to hydrogen sulfide, some of which are directly related to the inflammatory response (NFkB and Nrf2). These reports of direct hydrogen sulfide mediated effects are unfortunately few and far between and require more studies to confirm its validity as well as to define its implication on the inflammatory response. It is also subject to the question of specificity, if endogenous hydrogen sulfide is synthesised for a specific purpose, how does it reach its intended protein target without eliciting other effects? As a consequence, how could hydrogen sulfide modulating therapies do the same?

It is apparent at this stage that our current understanding of hydrogen sulfide is insufficient to warrant any therapeutic potential to be realised anytime soon. Despite this, the numerous studies showing hydrogen sulfide effect on the inflammatory response (Table 3) warrants more efforts into understanding and determining the direct effects which may offer potential therapeutic strategies. For example NFkB, an important pro-inflammatory transcription factor, has been shown to be s-sulfhydrated resulting in increased transcriptional activity. Monocytes/macrophages are shown to significantly increase CSE expression upon activation which could suggest the possible involvement of CSE/ endogenous hydrogen sulfide in the inflammatory function of these cells, possibly via modulation of NFkB activity. Assuming this is true, specific targeting of monocyte CSE activity/ endogenous hydrogen sulfide synthesis could help dampen the inflammatory response while still preserving the
function of these cells. A demonstration of this potential has been shown in *in vitro* siRNA knockdown studies of LPS stimulated macrophages (249, 250) whereby monocyte NFκB activation and pro-inflammatory cytokine release was reduced as well as in *in vivo* targeted knockdown of CSE in monocytes (251) where the inflammatory response was attenuated. However further studies are required to confirm the involvement of hydrogen sulfide and to determine the possible mechanism of its affect.

This would bring us to the third point of therapeutic window. Most of the pre-clinical studies including the monocyte targeted study above employ hydrogen sulfide intervention strategies before, during or shortly after disease onset. However, immunopathologies are generally associated with hyporeactivation and/or persistent activation of the immune response which may progress to an untimely deactivation (CARS) that may lead to subsequent susceptibility to secondary infections. Most patients are admitted when the disease have progressed and are possibly in the CARS state, therefore making the monocyte dampening therapy irrelevant. However there are incidences of immunopathologies where the window of opportunity is wider such as chronic inflammatory conditions like rheumatoid arthritis.

Therefore, with the current state of knowledge, it appears that hydrogen sulfide in itself does not possess or activate unique physiological effect or pathways respectively. However it appears to be able to modify certain protein activity and therefore exert indirect effects to known physiological processes. Therefore further insights into where hydrogen sulfide is being made, its targets and how that affects the inflammatory response could result in possible therapeutic strategies aimed at specifically attenuating or enhancing that hydrogen sulfide mediated pathway(s).

### 1.6 Acute pancreatitis

Acute pancreatitis is a common condition that accounts for more than 220,000 hospital admissions in the United States each year (252) with a mortality rate of 10% to 15% (253). A mild form of pancreatitis is characterized by localized self-limiting inflammation of the pancreas and resolves without the need of any intervention. Severe necrotizing pancreatitis is an uncontrolled form resulting in a systemic inflammatory response leading to multiple organ dysfunction and mortality primarily due to acute
respiratory distress. Following onset of a systemic inflammatory response, a compensatory anti-inflammatory response is initiated to regulate and dampen its effect. In certain cases, the hyperactivated state of the immune response during a systemic inflammation is followed by an excessive compensatory anti-inflammatory response which overly suppresses the immune system and facilitates nosocomial infections including infected pancreatic necrosis, one of the most feared complications of the disease. This makes treatment of acute pancreatitis difficult as the immunomodulation therapies aimed at dampening the initial hyperactivation of the immune response needs to be delivered in a timely fashion. The associated risk factors of acute pancreatitis are primarily gall stones and excessive alcohol intake (252), however, the precise aetiology of acute pancreatitis is still not completely understood. It is accepted that the pancreatic acinar cells of the exocrine pancreas plays a central role to the pathogenesis and progression of the ensuing inflammatory response. Currently, premature zymogen and NFκB activation in pancreatic acinar cells are believed to be the two major initiators (254).

1.6.1 Current understanding of acute pancreatitis aetiology

1.6.1.1 Premature zymogen activation

Uncontrolled premature zymogen activation results in autodigestion of the pancreas thus causing the release of intracellular contents rich in proteases and damage associated molecular pattern molecules (DAMPs) that elicit an inflammatory response. The mechanisms underlying premature zymogen activation have been the subject of intense study and has so far revealed shown to involve multiple events. Pathologic pancreatic acinar cell calcium influx is one; this entails a sustained global rise in cytosolic calcium influx (Ca$$^{2+}$$) as opposed to transient spikes observed in physiological responses (255). This response is reported in experimental models of pancreatitis and its blockade leads to inhibition of trypsinogen activation (256). Endoplasmic reticulum membrane Ryanodine Receptors (RyR) (257) and plasma membrane store operated calcium channels (SOCs) (258) have been implicated as important sources of Ca$$^{2+}$$. The Ca$$^{2+}$$ dependent activation of zymogens is thought to occur via calcineurin (a calcium dependent phosphatase) (259).
Other events leading to premature zymogen activation include the localisation of lysosomes and zymogens which allows lysosomal cathepsin B to cleave and activate zymogens (260, 261), however this requires additional conditions such as low pH which significantly enhances the catalytic activity of cathepsin B (262). The acidification of these vacuoles is thought to occur via vacuolar ATPase (vATPase) which pumps protons into the vacuoles (263). Finally, autophagy, although a protective response for cells has been shown to be both beneficial as well as deleterious in the progression of the disease in relation to premature zymogen activation (264, 265). The protective form of autophagy is thought to be more selective and involve zymogen granules thus termed zymophagy, the upregulation of zymophagy resulted in decreased trypsinogen activation and disease severity (265). In addition, the balance between lysosomal cathepsin B (activates trypsinogen) and cathepsin L (degrades trypsin) is also thought to affect the autophagy response as prematurely activated trypsinogen would undergo degradation by cathepsin L but during pancreatitis this is retarded due to imbalance between cathepsins B and L (266).

1.6.1.2 Activation of NFκB and the inflammatory response

NFκB is a known pro-inflammatory transcription factor that is highly activated at sites of inflammation in diverse diseases (199). Although both zymogen and NFκB activation are observed in pancreatitis, it has been shown that induction of pancreatitis in mice lacking trypsinogen-7, the critical enzyme required for zymogen activation, the disease still progressed without affecting the activation of pancreatic NFκB (267). This points strongly to the capacity of pancreatic cells in directly generating pro-inflammatory cues that subsequently elicit a full blown inflammatory response, independent of preceding zymogen activation. Current data suggests that pathologic Ca\(^{2+}\) (268) as well as activation of novel isoforms of protein kinase C (269-272) are responsible for NFκB activation. Mechanisms other than calcium or PKC dependent pathways have also been suggested, notable among these being angiotensin 2 acting through AT2 receptors (273) and p38/MAPK pathways (274, 275). Recently, the role of acinar cell injury by itself to activate inflammatory cascades was explored. It was found that cell injury may lead to release of damage-associated molecular pattern (DAMP) molecules such as high-mobility group box...
protein 1 (HMGB1), DNA, ATP, and heat shock protein 70 causing the activation of DAMP receptors (TLR-9 and P2X-7 recognized as important ones) (276). Activation of DAMP receptors induces formation of a cytosolic complex termed the inflammasome that is implicated in initiating inflammatory cascade during pancreatic injury (277).

1.6.1.3  **Bacterial infection – the second hit**

The initial injury that occurs in acute pancreatitis is characteristically sterile and involves the mechanisms described above (276). However, depending on the severity, the progression of acute pancreatitis is also associated with bacterial infection termed as the ‘second hit’. In severe acute pancreatitis, there is a disturbance in intestinal motility and permeability; ineffective motility leads to bacterial overgrowth in the small bowel and coupled with increased permeability promotes bacterial translocation through the intestinal mucosa resulting in bacteraemia and infections at remote sites (including peripancreatic or pancreatic necrosis) (278). Infected peripancreatic or pancreatic necrosis in patients with acute pancreatitis is associated with 30% mortality (279). The overall mortality in acute pancreatitis is about 5%, 50% of which is caused by infected necrosis (280) thus highlighting the importance of secondary bacterial infection as a cause for acute pancreatitis associated mortality.

Therefore intestinal function plays a pivotal role in the progression of this disease with regards to the secondary bacterial infection. There are currently multiple pathophysiological factors that are thought to contribute towards intestinal barrier dysfunction during pancreatitis. Of greater importance is possibly the reduction in intestinal blood flow (281) mediated by the vasoconstrictor endothelin (282), particularly that affecting the colonic mucosa leading to ischaemia and reperfusion injury thus leading to damage and disruption of the intestinal barrier (282, 283). Other factors include increased intestinal ICAM-1 expression leading to subsequent recruitment of leukocytes (284) as well as malnutrition that is associated with intestinal mucosal atrophy (285) and altered intestinal (286) and systemic immune defenses (287), which lead to an increase in intestinal permeability (288) with subsequent translocation of bacteria and endotoxin (289).
1.6.2 Acinar cell derived inflammatory mediators

Apart from releasing intracellular contents rich in DAMPs due to cell damage, intact pancreatic acinar cells are capable of actively synthesizing and releasing a spectrum of potent pro-inflammatory mediators that include chemokines, cytokines and substance P upon hyperstimulation. It is interesting to note that these cytokines and chemokines share a common transcriptional regulator, NFκB (290). In fact, NFκB has been shown to be the key regulator of increased cytokine and chemokine synthesis in acini in response to hyperstimulation and pancreatitis (291-293). While substance P expression is not shown to be regulated by NFκB, it has been shown upregulate chemokine expression and release in pancreatic acini via an NFκB mediated pathway (294). MCP-1 and MIP-2α are chemokines that have been shown to be upregulated and released by acini in response to pancreatitis (291, 293, 295). These chemokines recruit monocytes/macrophages to the site of injury, and in pancreatitis, peritoneal macrophages are thought to play an active role in propagating the inflammatory response following acinar cell injury (296, 297). Pro-inflammatory cytokines such as IL-1β, TNF-α and IL-6 have also been shown to be upregulated and released by acini in vivo and in vitro (292, 293). Substantial quantities of these cytokines have been detected as early as 30 mins in the pancreas (298) and 1 h in peritoneal fluid (300) of pancreatitic rats after induction. Together, these cytokines are able to activate and direct the progression of an inflammatory response by activating immune response cells, upregulating expression of endothelial cell adhesion molecules and inducing the acute phase protein response (301, 302). Substance P is a known mediator of neurogenic inflammation that is a potent chemoattractant, stimulates immune response cells, and promotes vascular permeability (303). Pancreatic acinar cells have been shown to increase substance P synthesis and release in response to caerulein hyperstimulation (304) and this is supported with evidence of increased pancreatic substance P levels in a caerulein model of acute pancreatitis (305). Substance P has also been shown to stimulate chemokine synthesis in pancreatic acini (294). Together, these mediators have been shown to contribute to the disease severity as evidenced by the protection afforded through genetic deletion of TNF-α receptor (306), IL-1β receptor (306), substance P (305) and pharmacological inhibition of MCP-1 (307). These mediators however were not associated with the development of the disease; this
would suggest that their role is more related to the ensuing inflammatory response of pancreatitis resulting in aberrant and uncontrolled recruitment and activation of immune cells.

### 1.6.3 Immune response cells in acute pancreatitis

Inflammatory mediators, though essential in the activation and progression of the inflammatory response are not directly responsible for the pathology observed in autoinflammatory diseases such as acute pancreatitis. The crucial effector element responsible for both the controlled eradication of invading pathogens as well as uncontrolled damage of healthy host tissue is the immune response cell. These cells possess the arsenal for potent and mass cell/tissue destruction through the harsh oxidizing chemicals and proteases that it is able to synthesize (refer to section 1.4). Macrophages and neutrophils are the predominant cell types involved in the inflammatory response of acute pancreatitis (308).

Due to their proximity, resident peritoneal macrophages are thought to be the key inflammatory cells to be initially activated and thus contribute to the inflammatory response in acute pancreatitis (297). Peritoneal macrophages are in direct contact with ascitic fluid secreted by the pancreas. This fluid generated in severe acute pancreatitis contains pancreatic enzymes and cytokines (300, 309). *In vitro* treatment of macrophages with peritoneal fluid harvested from pancreateitic rats resulted in activation of NF-kB and upregulation and secretion of TNF-α (310, 311). This activation has been shown to occur when specific pancreatic enzymes such as elastase, carboxypeptidase A and lipase come in direct contact with macrophages (312). Activated macrophages are then capable of synthesizing and secreting large quantities of inflammatory cytokines and chemokines (313) thus propagating the inflammatory response. In severe pancreatitis, neutrophils represent the largest subset of inflammatory cells present in the pancreas and peritoneal cavity (296, 300). Neutrophil infiltration increases over the course of the disease (300) and this is driven by the accumulating pro-inflammatory cytokines and chemokines that are synthesized and released by the inflamed pancreas and resident pancreatic and peritoneal macrophages (308).

Neutrophils are capable of causing more devastating damage than macrophages through the reactive oxygen species and proteases that they generate. Inhibition of polymorphonuclear cell (mostly
neutrophils) recruitment through use of ICAM-1 neutralising antibodies have shown substantial reduction in pancreatic MPO activity and oxidative damage, demonstrating the capacity of neutrophils to generate reactive oxygen species and directly contributing towards oxidative stress (314). Neutrophil depletion studies using anti-PMN serum has also shown substantial reduction of metalloprotease particularly in the gut followed by lowered bacterial translocation, highlighting the role of neutrophils in pancreatitis associated gut dysfunction (315).

Peritoneal monocyte/macrophage depletion prior to induction of pancreatitis was shown to lessen the systemic inflammatory response as evidenced by lowered peritoneal and serum cytokines, lowered neutrophil infiltration and lung injury (296). However it was noted that there was no change in pancreatic injury, this suggests that the peritoneal macrophages have an important role to play in propagating the inflammatory response but not in the pathogenesis of severe pancreatitis. A similar finding was observed in neutrophil depletion studies with substantial lung protection and diminished mortality (316, 317). This highlights the importance of the innate inflammatory cells in pancreatitis, particularly in the propagation and execution of the uncontrolled inflammatory response.
Figure 1.9 The inflammatory response associated with acute pancreatitis. The precise aetiology of acute pancreatitis is still not clearly understood but it is known to involve premature activation of zymogens and activation of NF-κB in pancreatic acinar cells. This leads to the uncontrolled release of digestive enzymes that serve as DAMPs as well as the synthesis and release of pro-inflammatory mediators such as cytokines, chemokines and eicosanoids. These mediators are released into the peritoneal cavity resulting in the activation and recruitment of resident pancreatic and peritoneal macrophages thus initiating the cascade of innate inflammatory response and the recruitment of neutrophils. Together, these inflammatory cells synthesize and contribute to the accumulation of a large amount of pro-inflammatory mediators such as ROS, proteases, chemokines, cytokines and eicosanoids in the ascitic fluid of the peritoneal cavity. This fluid is then channelled into the lymphatic system and drains into the circulation via the thoracic duct into the left subclavian vein resulting in a systemic inflammatory response. The lungs are the first organ to be bathed with these inflammatory mediators and it acts as a sponge to soak it up with its extensive network of vasculature leading to lung injury, the leading cause of mortality associated with this disease.

1.6.4 Effect of endogenous hydrogen sulfide in pancreatitis

So far, there have been several but limited in vivo and in vitro studies on the effect of endogenous hydrogen sulfide in pancreatitis. All of these studies have employed the use of PAG as a pharmacological inhibitor of endogenous hydrogen sulfide synthesis and in doing so targeted CSE
activity specifically. Although PAG is selective towards CSE among the hydrogen sulfide synthesizing enzymes, it is also known to inhibit activity of other enzymes (see section 1.5.4). Both *in vivo* and *in vitro* studies corroborate well showing that CSE expression plays a deleterious role in pancreatitis (66, 304, 318, 319). So far there has been one report showing increased pancreatic acini CSE expression and hydrogen sulfide synthesizing activity in response to caerulein stimulation *in vitro* (304); inhibition of CSE resulted in a reduction of the classic signs associated with pancreatitis such as acinar cell injury, plasma amylase and pancreatic neutrophil infiltration (as evidenced by MPO activity) *in vivo* (66). *In vivo* studies have attributed this protection to a reduction in pancreatic chemokines (318), potent drivers of the inflammatory response (see section 1.6.1.3), and this is supported by *in vitro* findings using caerulein stimulated pancreatic acini (318). Additional *in vitro* findings showed a CSE mediated upregulation and expression of pancreatic acini adhesion molecule, ICAM-1, in response to caerulein stimulation via activation of NFκB and Src family kinases (319). Lastly, elevated levels of pancreatic substance P, a known neurogenic inflammatory mediator (see section 1.4.4.1), was attenuated following CSE inhibition in pancreatitis (244) and this was also observed *in vitro* (304). Taken together, these PAG inhibitor studies have established a pro-inflammatory role for CSE/endogenous hydrogen sulfide in pancreatitis. Current findings suggest an important role for CSE enzymatic products in the activation of NFκB and downstream inflammatory mediators such as MIP-2α, MCP-1 and ICAM-1 in the pancreas. This CSE mediated activation of NFκB in turn is thought to occur via substance P. There is however still limited studies on *in vivo* CSE expression in the progression of pancreatitis and its effect on other inflammatory mediators such as cytokines and eicosanoids.

Another aspect of hydrogen sulfide effect on the exocrine pancreas that has not been studied in the context of pancreatitis is its ability to increase pancreatic acini intracellular calcium levels (320). There was a dose dependent biphasic increase with an initial rapid rise followed by a long-lasting elevation. This was found to be mediated by hydrogen sulfide-evoked nitric oxide production via an endothelial nitric oxide synthase-nitric oxide soluble guanylate cyclase-cyclic guanosine monophosphate-protein kinase G-Gq-protein-phospholipase C-inositol 1,4,5-trisphosphate pathway to induce calcium release. As mentioned in section 1.6.1.1, persistent increase in acini intracellular calcium is associated with premature zymogen activation, a major initiating factor in acute pancreatitis. Therefore increases in
endogenous hydrogen sulfide levels could contribute to the progression of the disease. However, there are no published reports demonstrating this link at present.

Currently, reports on the role of CSE/endogenous hydrogen sulfide in pancreatitis have been focused on its effect in the pancreas and the pancreatic acini and none on the effect in immune response cells, particularly the innate response cells. Though they may not be directly involved in the aetiology of the disease, they are the key effector cells of the inflammatory response (see section 1.5.1 and 1.6.3), therefore understanding the effect of CSE/endogenous hydrogen sulfide in these cells could not only offer insights into pancreatitis but the inflammatory response in general. Thus far, there have been several but limited studies looking at the role of CSE/endogenous hydrogen sulfide in innate response cells, macrophages in particular. Oh et al were the first to show that CSE is expressed in macrophages and was upregulated upon LPS activation (201) and later corroborated by Zhu et al (55). Both studies showed a CSE/endogenous hydrogen sulfide downregulation of iNOS expression and NO production in LPS activated macrophages by hydrogen sulfide mediated through an NFκB-heme oxygenase/CO dependent pathway. This anti-inflammatory effect of CSE/endogenous hydrogen sulfide was also shown in oxidized-LDL (ox-LDL) activated macrophages; basal CSE/endogenous hydrogen sulfide was shown to inhibit NFκB activation followed by subsequent cytokine and chemokine synthesis and oxidized-LDL treatment resulted in downregulation of CSE expression causing activation (205, 321).

Interestingly, there have also been reports suggesting a pro-inflammatory role of CSE/endogenous hydrogen sulfide instead in LPS stimulated macrophages resulting in increased production of pro-inflammatory cytokines via an NFκB dependent pathway (249, 250). These conflicting findings could be influenced by macrophage plasticity which allows these cells to transition between a pro and anti-inflammatory state (297). It is still early days in the research on CSE/endogenous hydrogen sulfide in immune cells and more studies are required to paint a clearer picture on the role(s) of CSE/endogenous hydrogen sulfide in the activation and regulation of these cells. In relation to pancreatitis, it is still yet to be determined if there is a dysregulation of CSE expression in the immune cells and what its effect may be.
The role of CSE/endogenous hydrogen sulfide in pancreatitis. The illustration on the left shows the current reported effects of CSE expression/endogenous hydrogen sulfide in pancreatic acinar cells following caerulein hyperstimulation. Caerulein stimulation has been reported to increase CSE expression in pancreatic acinar cells leading to increased expression of substance P and activation of NFκB. Substance P is a known neurogenic inflammatory mediator and has been shown to elicit pancreatic acinar cell chemokine release. NFκB is a transcription factor for pro-inflammatory genes, CSE mediated activation of NFκB was linked to increased expression of the adhesion molecule ICAM-1. These effects were abrogated by pre-treating cells with PAG, a CSE inhibitor. In vivo CSE inhibition also showed a protective effect in response to pancreatitis in mice. There was attenuation in disease severity as evidenced by lowered pancreatic and lung leukocyte infiltration as well as acinar cell injury. This was attributed to a reduction of pancreatic pro-inflammatory mediator production, namely chemokines and cytokines.

1.7 Current knowledge and gaps on the effect of CSE/endogenous hydrogen sulfide in acute pancreatitis and the inflammatory response

1.7.1 Specificity of CSE inhibition in in vivo models of inflammatory disease

CSE has been shown to be the key inflammatory modulating hydrogen sulfide synthesizing enzyme in animal models of disease (1-12). So far, studies aimed at elucidating the role of CSE in inflammation have mostly employed the use of PAG (1-6,8-15) and to a lesser extent, siRNA in in vitro systems (16,17). However as mentioned in section 1.4.3 there is still little agreement as to the actual role of hydrogen sulfide in inflammation and the use of PAG has been highlighted as one of the possible contributing factors due to its non-specific inhibitory activity (reviewed by (243)). Therefore alternative methods of CSE inhibition such as the use of CSE deficient animals would highly useful in affirming the
role of CSE in inflammation by avoiding any non-specific effects of pharmacological inhibitors like PAG. At the time this thesis was conceived and carried out there had been no reports on the use of CSE deficient animals in models of inflammatory disease.

1.7.2 The effect of CSE in acute pancreatitis

Acute pancreatitis is a form of sterile inflammatory disease in its less severe form, and therefore a useful model to study the inflammatory response independent of pathogenic effects; this is particularly useful in the field of hydrogen sulfide whereby bacteria are capable of producing hydrogen sulfide which in turn also affects them. To date, all published studies with regards to the role of hydrogen sulfide in acute pancreatitis using PAG have shown CSE to be pro-inflammatory (66, 69, 318, 322). CSE/endogenous hydrogen sulfide has been shown to mediate the expression of key inflammatory mediators in the pancreas; MCP-1, MIP-2α, TNF-α and NFκB (301, 307).

1.7.2.1 CSE and eicosanoids in acute pancreatitis

There are currently no reports on the effect of CSE/endogenous hydrogen sulfide on eicosanoids, a potent modulator of the immune response (323), in acute pancreatitis. Eicosanoids are small lipid based signalling molecules comprising of prostaglandins and leukotrienes that are regulated mainly through the action of two enzymes cyclooxygenase-1 and cyclooxygenase-2. These molecules are able to direct the progression of the inflammatory response by modulating vascular permeability, cell adhesion as well as chemoattraction and activation of leukocytes (324). Therefore measuring acute pancreatitis mediated changes in eicosanoid levels in relation to CSE expression would provide additional insight into the role of CSE/ endogenous hydrogen sulfide in the progression of the disease as well as contribute towards the current understanding of CSE effect in the inflammatory response at large.

1.7.2.2 CSE dysregulation in acute pancreatitis

Although CSE dysregulation has been reported in various models of disease (44, 45, 189, 223, 325-330), there was no direct evidence of change in pancreatic CSE expression in acute pancreatitis or the
determination of the source at the early phase of this thesis. Also, there currently aren’t many studies looking at CSE dysregulation in macrophages, the drivers of the innate inflammatory response. All of these studies involve in vitro stimulation with single purified molecules such as LPS and ox-LDL (55, 201, 205, 249, 250, 321) and there are currently no reports linked to in vivo disease models of inflammation. Demonstration of macrophage CSE dysregulation in response to an in vivo inflammatory stimuli would further strengthen its role in the function of these immune cells at therefore the inflammatory response at large.

1.7.2.3 Correlating CSE dysregulation with changes in endogenous hydrogen sulfide levels and elucidating the direct targets of hydrogen sulfide

Since the discovery of hydrogen sulfide role in inflammation, there have been a great number of studies looking at the downstream effects of endogenous hydrogen sulfide synthesized by CSE in association with known inflammatory events such as cytokine and eicosanoid synthesis (14,18,19), regulation of adhesion molecules expression (10,13,20), and immune cell modulation (16,17) to name a few. However there is little information on the mechanisms leading up to CSE overexpression, its correlation with endogenous hydrogen sulfide levels and the mechanism by which hydrogen sulfide would directly affect the inflammatory response. Therefore by correlating CSE dysregulation with changes in endogenous hydrogen sulfide levels and elucidating the mechanism by which it could directly affect the inflammatory response, this would greatly support currently claims of the role of CSE/ endogenous hydrogen sulfide as an inflammatory mediator.

Figure 1.11 illustrates the state of current knowledge and gaps.
Figure 1.11 Illustration of current knowledge on CSE/endogenous hydrogen sulfide effect on acute pancreatitis and macrophages as well as aims of this thesis. CSE/endogenous hydrogen sulfide has been shown to play a pro-inflammatory role in acute pancreatitis through pharmacological inhibition studies using PAG, however there are limitations to using PAG namely its non-specific inhibitory effect. Therefore using mice genetically deficient in CSE would be a useful alternative method. In vitro studies have shown macrophages to upregulate CSE expression in response to LPS or ox-LDL stimulation and this resulted in both pro and anti-inflammatory effects. In acute pancreatitis, there has been indirect evidence of increased pancreatic CSE expression by measuring hydrogen sulfide synthesizing activity. It would therefore be worthwhile to directly determine if pancreatic CSE is upregulated following pancreatitis as well as investigate the effect of the disease on macrophage CSE expression status and the possible pathways that lead to CSE dysregulation in macrophages. Finally it would be interesting to correlate possible dysregulation of CSE observed in acute pancreatitis and macrophages with changes in protein s-sulfhydration state, a potential means of direct hydrogen sulfide mediated effect.

1.8 Aims of this thesis

I have investigated the role of endogenous hydrogen sulfide synthesised by CSE in the inflammatory response using acute pancreatitis as the disease model. My hypotheses were that CSE contributes to the inflammatory response in acute pancreatitis by increasing endogenous hydrogen sulfide synthesis and genetic deletion of CSE would lessen the severity of acute pancreatitis and the associated inflammatory response. The overall aim is to validate previous reports on the inflammatory
role of CSE/ endogenous hydrogen sulfide in acute pancreatitis by means of genetic deletion instead of pharmacological inhibition and to investigate the relevance of CSE expression in the immune response. The specific aims are shown below with a brief outline following:

1) To refine an existing simple high-throughput method that would fulfil the criteria for specific and sensitive detection of free hydrogen sulfide in tissue with standard laboratory equipment.

2) To correlate CSE dysregulation with changes in hydrogen sulfide mediated protein modifications (s-sulfhydrated proteins) as an indicator or footprint of CSE mediated hydrogen sulfide synthesis.

3) To determine changes in CSE expression in the pancreas and macrophages in response to acute pancreatitis, and the molecular mechanisms involved.

4) To determine the effect of CSE on the inflammatory response and disease severity using mice genetically deficient in CSE as an alternative to pharmacological inhibition.

1) To refine an existing simple high-throughput method that would fulfil the criteria for specific and sensitive detection of free hydrogen sulfide in tissue with standard laboratory equipment

   - This work was based on a zinc precipitation of hydrogen sulfide and wash technique by Gilboa-Garber (21). An alkaline-zinc reagent was used to simultaneously precipitate free hydrogen sulfide and keep proteins in solution without releasing endogenous acid labile sulfide. The ZnS precipitate was then separated, washed, and the amount of sulfur present measured.

2) To determine changes in CSE expression in the pancreas and macrophages in response to acute pancreatitis, and the molecular mechanisms involved.

   - Investigated the biotin-switch (22) and tag-switch (23) methods of detecting s-sulfhydrated proteins as a way of measuring changes in protein s-sulfhydration levels.
3) To determine changes in CSE expression in the pancreas and macrophages in response to acute pancreatitis, the molecular mechanisms involved.

- For the in vivo acute pancreatitis studies, pancreatic CSE expression levels were measured by western blot and immunohistochemistry. Immunohistochemistry also served to determine the location/cell type where this occurs.
- For the macrophage CSE expression in vitro studies, LPS, a known activator of macrophages was used as a positive control to optimise methods of CSE expression measurement by western blotting and quantitative PCR as well as the concentration of pharmacological inhibitors used to elucidate the underlying molecular mechanisms.
- Acute pancreatitis associated macrophage CSE expression was then determined by in vitro stimulation with plasma of pancreatitisic mice using the optimised methods established above. Prior to this, the activation of macrophages with plasma of pancreatitisic mice was analysed by measuring pro-inflammatory cytokine and chemokine secretion in order to establish its relevance.

4) To determine the effect of CSE on the inflammatory response and disease severity using mice genetically deficient in CSE as an alternative to pharmacological inhibition

- The effect of CSE deficiency was studied using an in vivo murine model of caerulein induced acute pancreatitis as well as in vitro using primary pancreatic acini stimulated with caerulein to induce a pancreatitis like state.
- Disease severity was determined through the classical pathophysiological events associated with acute pancreatitis; increased plasma amylase, increased pancreatic and lung MPO activity (a sign of neutrophil infiltration), pancreatic and lung oedema as well as acinar cell damage
- The inflammatory response was assessed by measuring NF-κB activation as well pro-inflammatory mediators such as cytokines, chemokine and eicosanoids in the pancreas from in vivo experiments and in the supernatant of pancreatic acini from in vitro experiments

As a result of this thesis, I hope to fill in the gaps in the current knowledge of the role of CSE/endogenous hydrogen sulfide on acute pancreatitis and macrophages as illustrated in Figure 1.11.
2 Measuring tissue hydrogen sulfide content and protein s-sulphydration

2.1 Introduction

2.1.1 Measuring endogenous free tissue sulfide

The detection and measurement of endogenous levels of bioactive molecules provide researchers with vital information of its relevance to certain physiological or pathophysiological events. A measured increase or decrease in response to a particular stimulus would establish a strong correlative role in line with the purpose of the study. Additionally, accurate estimates of endogenous levels would provide a more physiologically relevant framework for in vitro studies in the attempt to elucidate the role of these molecules that would in the longer term translate to in vivo findings.

The ability for endogenous hydrogen sulfide synthesis was first demonstrated by Stipanuk et al (1982) (11) using the methylene blue method of detecting free hydrogen sulfide. Briefly, the method involves the initial trapping of free sulfide by zinc acetate to form an insoluble zinc sulfide (ZnS) precipitate and in contact with an oxidizing agent such as ferric chloride in a strongly acid solution, it reacts with the N,N-dimethyl-p-phenylenediammonium ion to yield methylene blue that is readily detectable by spectrophotometry with a maximum spectra at 667nm and coefficient constant of 71,090 M$^{-1}$ cm$^{-1}$ (34). The overall reaction can be summarised as below:

This reaction is thought to involve the initial oxidation of N,N-dimethyl-p-phenylenediamine by Fe$^{3+}$ to form the following intermediate (331):
The intermediate is then reduced by hydrogen sulfide to form the phenotiazinium dye, methylene blue (331):

\[ \text{N,N-dimethyl-p-phenylendiamine} \rightarrow \text{Wurster's red} \]

A more detailed reaction scheme has been described by Kuban et al (332) as shown in Figure 2.1. The initial step requires the oxidation of N,N-dimethyl-p-phenylendiamine to form a radical cation (Figure 2.1a), this reaction is highly facile and the stable product is known as Wurster's red (333). This is followed by the addition of hydrogen sulfide to the cation-radical and further electron transfer, as shown in Figure 2.1b. The intermediate mercapto derivative of N,N-dimethyl-p-phenylendiamine would then add to a second cation radical, followed by further oxidation, to yield the sulfide-bridged dimer (Figure 2.1c). A third 2-electron oxidation sequence, followed by addition of the amino group to the imine functionality, favoured by proximity (Figure 2.1d), with subsequent loss of ammonia (Figure 2.1e) would complete the synthesis. If hydrogen sulfide was substituted with a mercaptan, the second addition of step (c) in Figure 2.1 is blocked. Unlike methylene blue, the mercaptan products is thought to be an S-alkonium phenothiazinium dication and the spectral characteristics of the orange-red mercaptan products (\(\lambda_{\text{max}} 490-510\text{ nm}\)) are very different from those of methylene blue (334).
Figure 2.1 Detailed reaction scheme for generation of methylene blue from N,N-dimethyl-p-phenylenediamine and hydrogen sulfide under oxidising conditions with iron(III)chloride. (a) Oxidation of N,N-dimethyl-p-phenylenediamine by Fe$^{3+}$ to form a stable cation radical, Wurster’s red (b) Addition of hydrogen sulfide to the cation-radical and further electron transfer (c) Addition of mercapto derivative of N,N-dimethyl-p-phenylenediamine intermediate in step (b) to a second cation radical, followed by further oxidation to yield the sulfide-bridged dimer (d) A 2-electron oxidation sequence, followed by addition of the amino group to the imine functionality, favoured by proximity (e) Subsequent loss of ammonia to yield methylene blue.
The methylene blue assay is simple to perform with the requirement of some basic chemicals and the use of a standard spectrophotometer. As such, it has been widely used in the detection of free sulfide in biological solutions such as plasma (12, 18, 44). Early studies showed substantial micromolar levels of free endogenous hydrogen sulfide (12, 32, 35, 42, 43) that established the notion of its physiological significance leading to an exponential growth in hydrogen sulfide studies with regards to its diverse physiological and pathophysiological roles. However, it was not until recently that these high levels were suggested to be an overestimate possibly due to labile sulfide that is released. This was thought to be the result of the highly acidic conditions of the methylene blue assay thus releasing bound acid-labile sulphide (25, 29). Other criticisms of the methylene blue is that the absorbance does measured at 667nm does not obey Beer’s law, however it was shown that this can be avoided with the use of an appropriate ratio of N,N-dimethyl-p-phenylenediamine and iron(III)chloride to sulfide to achieve an optimal stoichiometry (71). Several other methods of detection have been employed since the initial hydrogen sulfide studies; these include the use of polarographic electrodes (32, 54), gas chromatography (37, 41), liquid chromatography with monobromobimane derivitization (36)], and hydrogen sulfide specific fluorescent probes (49, 50, 61, 335) (further elaborated in section 1.2.4).

The development of newer methods of detection have in principal improved the levels of detection as well as increased specificity, however there are still some drawbacks or limitations to these methods. The polarographic sensor though specific and sensitive has very low through put, is only able to detect the gaseous hydrogen sulfide form and in the current set up is suited mainly to measure hydrogen sulfide synthesis. The specificity and sensitivity of fluorescent probes have increased over time, however there is the issue of background fluorescence in tissues and plasma that has to be accounted for. Recent methods have tried to account for this by generating internal standards using spiked hydrogen sulfide into the homogenate or plasma of study and taking the difference with zinc precipitated sulfide samples as the zero reference (48-50). However the reported values were a few orders off from the gas chromatography and monobromobimane/ RP-HPLC method described by Levitt et al and Shen et al respectively (36, 37). The Levitt et al and Shen et al methods appear to be the best methods so far which takes into account hydrogen sulfide stability during sample preparation and employs methods that are highly sensitive and specific. However both of these methods require the use of specialised instruments that are not commonplace in most laboratories. Furthermore the level
of throughput is not high particularly with the gas chromatography method described by Levitt et al. In contrast, the spectrophotometric methylene blue assay that is still widely used is simple and of high throughput though it suffers from heavy criticism due to the harsh acidic conditions of the assay which could liberate endogenous acid labile sulfur.

Despite the development of newer methods of endogenous hydrogen sulfide detection such as the polarographic sensor, fluorescent probes, gas chromatography and monobromobimane derivatisation/HPLC, there does not seem to be a consensus among researchers to favour or adopt a particular method of detection. In fact apart from the monobromobimane derivatisation/HPLC method which has been used and cited 6 times (46, 47, 72, 73, 75, 76), none of the other methods have been used (cited) much or at all to measure endogenous levels of hydrogen sulfide. The polarographic sensor has been used mainly to measure \textit{ex vivo} or \textit{in vitro} hydrogen sulfide synthesis (55). There are currently no original research articles that have used or cited the use of fluorescent probes to measure endogenous levels of hydrogen sulfide. The gas chromatography method developed by Levitt et al has not been used or cited. However there are other gas chromatography methods that have been used although with a slightly different protocol, one under highly acidic conditions that could potentially liberate acid labile sulfur (328) and the other two had samples homogenised at pH 7.4 which could result in loss of gaseous hydrogen sulfide during preparation (40, 41). It would be interesting to note that these gas chromatography based methods also used a sulfur chemiluminescent detector but have all reported free tissue hydrogen sulfide levels that are 2 to 6 orders higher than that reported by Levitt et al. Despite its limitations and the main criticism of overestimating levels of endogenous free hydrogen sulfide (presumably via liberation of acid labile sulfide), the spectrophotometric method remains a popular method of measurement (67-69). The review by Kolluru et al (214) provides an excellent comparison of the advantages and disadvantages of these methods and this is summarised in Table 4.

<table>
<thead>
<tr>
<th>Method</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Ref</th>
</tr>
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<tbody>
<tr>
<td>Polarographic sensor</td>
<td>- Sensitive detection (nM)</td>
<td>- Low through-put</td>
<td>(53)</td>
</tr>
<tr>
<td>(hydrogen sulfide electrode)</td>
<td>- Real time detection</td>
<td>- Detects only hydrogen sulfide species</td>
<td></td>
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<td></td>
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<td>- Used to measures synthesis rate and</td>
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Table 4 Advantages and disadvantages of various recent methods of detecting endogenous levels of free hydrogen sulfide. This table provides an account for the comparison of current methods developed and used to measure endogenous levels of hydrogen sulfide based on their advantages and disadvantages.

Methylene blue method, despite its own limitations, continues to be a popular method to detect endogenous levels of hydrogen sulfide (69, 336-341). Possible reasons for the poor reception of these apparently improved methods and continued use of the methylene blue assay in the detection and quantification of endogenous hydrogen sulfide could be due to the specialised equipment required that is not commonplace and that they are not standard laboratory techniques. This is an area of concern that needs to be addressed. In the initial period of my Phd, I did not have access to a gas or liquid chromatography instrument and therefore sought improvise the current spectrophotometric method of detecting hydrogen sulfide using methylene blue. The main aim was to improve specificity
towards free hydrogen sulfide by circumventing the harsh acidic conditions of the assay as well as to improve the sensitivity. If successful, this method could be more readily adopted and utilised in the field of hydrogen sulfide as a means of measuring endogenous levels of free hydrogen sulfide.

2.1.2 Hydrogen sulfide mediated s-sulfhydration (persulfide formation)?

There has been numerous studies demonstrating the (patho)physiological effects of endogenous and exogenous hydrogen sulfide as described in section 1.3. However there are few that show the direct mechanism by which hydrogen sulfide exerts its effect, in other words the target effector molecules of hydrogen sulfide. To date there are 3 methods by which hydrogen sulfide has been reported to do so; as an antioxidant, through s-sulfhydration (formation of protein persulfides) and binding to metal ions/ metal ion containing proteins (elaborated further in section 1.4). There has been much contention on the detection of free hydrogen sulfide levels partly due to the transient and highly reactive nature of hydrogen sulfide. This led us to speculate that the measurement of protein persulfide levels could serve as a signature or indicator of hydrogen sulfide levels instead. In doing so, we would also be able to determine the effector molecules of hydrogen sulfide and further our understanding of the mechanisms behind hydrogen sulfide mediated effects.

I chose to pursue a method of protein persulfide detection that would allow visualisation and comparison of global protein persulfide levels. The intention was to use this method to determine if there were changes in protein persulfide levels under inflammatory conditions of acute pancreatitis which is the subject of study in chapter 5. The reason for measuring global levels is due to the uncertainty of which protein might undergo persulfidation changes and it would be costly to target multiple specific proteins. I elected to use a modified biotin-switch technique which was initially developed to detect s-nitrosylated proteins (342) and modified for this purpose (31). This method is reported to detect and semi-quantitate global levels of s-sulfhydrated proteins by probing for biotin labelled proteins on a western blot making it an appealing choice for assessing overall changes in endogenous sulfhydration state. It was developed and employed by Mustafa et al and was the first report showing the ability of hydrogen sulfide to directly s-sulfhydrate proteins under physiological conditions (31). In this modified biotin switch technique, thiols are first alkylated with methyl methanethiosulfonate (MMTS),
and s-sulphydrated proteins (persulfides) were postulated to remain unreacted and be available for subsequent conjugation to N-[6-(biotinamido) hexyl]-3’-(2’-pyridyldithio) propionamide (biotin-HPDP).

Concerns over the chemistry behind this biotin switch method proposed by Mustafa et al 2009 (31) was later raised with regards to the use of MMTS as the selective thiol (R-SH) blocking agent which was thought to not react with persulfides (R-S-SH), it had later been shown that MMTS has similar reactivity towards nucleophiles such as thiols and persulfides (343). During the course of my thesis, another research group (Zhang et al 2014 (344)) had proposed a tag-switch technique that was reported to address the non-specific of MMTS blocking. They employed the use of a thiol blocking agent (MSBT) that would bind to both thiols and persulfides,

this was followed by a tag-switching reagent, CN-biotin (containing both the nucleophile, cyanoacetate and a reporting molecule, biotin) to label only the persulfide adducts.
Thiol adducts from the reaction of R-SH and MSBT are thioethers, which are not expected to react with the nucleophile cyanoacetate. In this thesis I had initially used the biotin switch technique to detect endogenous levels of protein s-sulfhydration as well as the biotin-tag switch method subsequently. An illustration and comparison of these methods are shown in Figure 2.2.
Figure 2.2 Illustration of the Biotin switch and Biotin tag-switch methods of detecting s-sulfhydrylated proteins. Both free thiol (-SH) and persulfide (-S-SH) groups have similar chemical properties making it difficult to distinguish them. This figure describes two methods of labelling protein persulfides with biotin tags for subsequent detection using western blot with streptavidin-HRP probes. The Biotin switch method (31) entails the use of a thiol blocking agent (MMTS) and subsequent labelling of free persulfides with biotin tagged HPDP. The Biotin tag-switch method (344) uses a thiol blocker (MSBT) that binds to both free thiols and persulfides, this is followed by labelling of MSBT blocked persulfides with biotin tagged cyanoacetate (CN-biotin).
2.2 Aims

The initial aim of this chapter was to develop a robust and high throughput method of detecting endogenous free hydrogen sulfide in tissues that should be technically simple and not require the use of highly specialised equipment.

The method was based on a zinc precipitation of hydrogen sulfide and wash technique by Gilboa-Garber (345). Tissue homogenates were treated with an alkaline-zinc reagent that would simultaneously precipitate free hydrogen sulfide and keep proteins in solution. This would facilitate the separation of hydrogen sulfide (in the form of an insoluble ZnS precipitate) from the rest of the homogenate which contains labile sulfide by centrifugation. The ZnS pellet would then be washed with water to remove residual contaminants. The detection of sulfide could then be performed using any desirable method; in this case methylene blue was used requiring the use of a standard spectrophotometer.

Additionally, this chapter will also investigate published methods of detecting protein s-sulfhydration in an attempt to compare protein s-sulfhydration state between tissues of wild-type and CSE deficient mice as well as to correlate changes in CSE expression with protein s-sulfhydration state as an indicator or footprint of CSE mediated H₂S synthesis.

2.3 Materials and methods

2.3.1 Materials

N,N-dimethyl-p-phenylenediamine dihydrochloride (NNDP) (Sigma) was prepared in a 7.2 N HCl solution. Iron(III)chloride (FeCl₃) (Merck) was prepared in a 1.2 N HCl solution. Na₂S nonahydrate (Sigma) is known to contain impurities which can interfere with sulfide measurement. Therefore, before use, Na₂S crystals were washed with water and dried under vacuum (34). These crystals were then dissolved and diluted into buffer in polypropylene microfuge tubes. Trichloroacetic acid (TCA), sodium hydroxide (NaOH) and zinc acetate were obtained from Merck.
2.3.2 The methylene blue assay without zinc precipitation for determination of sulfide levels ('current')

The methylene blue assay has previously been used to determine sulfide levels in biological solutions (44, 189). This assay was labelled as the ‘current’ method for convenience and clarity. In this method, 500 µL of a sample (Na₂S standard solution or tissue homogenate) was mixed with 350 µL of zinc acetate (1% w/v), 133 µL of 20 mM NNDP, 133 µL of 30 mM FeCl₃ and 250 µL of TCA (10% w/v). The mixture was then incubated for 10 min at RT to allow the colour to develop, before centrifuging at 15,000 g for 10 mins. The clear supernatant was read for absorbance on a spectrophotometer at 670 nm (Molecular Devices, Spectramax M5).

2.3.3 The methylene blue assay with zinc precipitation for the determination of sulfide levels ('modified')

The zinc precipitation methodology was based upon a modified protocol that was originally developed by Gilboa-Garber (345). In this thesis, this assay will be labelled as the ‘modified’ method for convenience and clarity. 500 µL of a sample (Na₂S standard solution or tissue homogenate) was added to 400 µL of a pre-mixed 1% w/v zinc acetate (350 µL) and 1.5 M sodium hydroxide solution (50 µL). This was followed by centrifugation at 1200 g (using a swing-out-rotor) for 5 min to pellet the ZnS that had been formed. The supernatant was then aspirated off and the pellet washed with 1.5 mL of Milli-Q water by vortexing thoroughly, followed by a centrifugation at 1200 g for 5 min. The supernatant was then aspirated off and the pellet reconstituted with 160 µL of Milli-Q water. At this point, the detection of sulfide present as ZnS within the pellet could be performed by any means chosen. The methylene blue method was chosen for its simplicity and ability to be read on a standard spectrophotometer. To do so, the ZnS suspension was mixed with 40 µL of pre-mixed dye (20 µL of 20 mM NNDP & 20 µL of 30 mM FeCl₃). The solution was then incubated for 10 min to allow the color to develop, before the absorbance was read on a spectrophotometer at 670 nm.
2.3.4 Determination of the effects of pH on the stability of sulfide solutions

For the pH stability studies, a 100 μM Na₂S standard solution was prepared in 50 mM sodium phosphate buffer at pH 6, 7, 7.5, 8, 8.5, and in 50 mM sodium carbonate buffer at pH 9 and 10. The tubes were then incubated for the designated time intervals at room temperature. To determine sulfide concentrations at each time point, 100 μL aliquots of the 100 μM Na₂S standard solutions were transferred to individual tubes and sulfide levels were fixed by the addition of 60 μL zinc acetate (1% w/v). After the final time point, 40 μL of pre-mixed dye (20 μL of 20 mM NNDP & 20 μL of 30 mM FeCl₃) was added to each tube and incubated for 10 min for the color to develop before reading on a spectrophotometer at 670nm.

2.3.5 Comparison of the original and modified methods for detecting sulfides

A series of standard Na₂S solutions were prepared using serially diluted Na₂S dissolved in 50 mM sodium carbonate buffer at pH 9. The sulfide content of each standard was then independently evaluated using both the original and modified assays. Additionally, to determine if zinc precipitation was effective in detecting sulfide in biological material, a standard curve was prepared using Na₂S added to liver homogenate (40 mg/mL) and processed using the modified method. A set of Na₂S standards was prepared in a 25 μL serial dilution to yield a final concentration of 0 to 8.33 μM in 500 μL when buffer or liver homogenate is subsequently added. These sets of Na₂S standards were precipitated with 400 μL of the zinc acetate and NaOH mixture, followed by addition of 500 μL liver homogenate or buffer alone and processed as described above either by the original or modified method.

2.3.6 Measuring tissue sulfide using the modified method

Use of animal tissues for this work was approved by University of Otago Animal Ethics committee (Protocol no 57/11). Tissues were harvested from 6 BALB/c mice euthanized with pentobarbital and immediately frozen on dry ice and stored at -80°C. On the day of experiment, 40 mg of tissue was weighed out and immediately immersed in 1 mL of buffer (described below). Samples were then homogenized and sonicated on ice followed by centrifugation at 15,000 g for 10 min at 4°C. 500 μL of
the clear supernatant was then used for the zinc precipitation assay as described. To determine free sulfide levels, a 40 mg piece of each tissue was prepared (as described above) in a 50 mM sodium phosphate buffer pH 6 and a 50 mM sodium carbonate buffer pH 9. Based on the reported hydrogen sulfide dissociation constant (6), at pH 6, >90% of free sulfides should exist as the neutral hydrogen sulfide species which is highly volatile and easily lost by dissipation into the atmosphere. At pH 9, >90% of free sulfides should exist as the anionic HS\(^{-}\) which is retained in solution. Free tissue sulfide was then calculated by deducting the value of total sulfides measured at pH 6 from pH 9.

In one set of experiments, a pH titration was performed to determine if the amount of measured sulfides in tissue adhered to the expected dissociation constant of hydrogen sulfide. This experiment was carried out with mice livers processed in 50 mM sodium phosphate buffer at pH 6, 6.5, 7, 7.5, 8 and 50 mM sodium carbonate buffer pH 9 using the method described above.

### 2.3.7 Free sulfide scavenging in a biological solution

The modified method was used to detect free sulfide in liver homogenates that were spiked with a known amount of Na\(_2\)S before or after the addition of the zinc acetate and NaOH mixture to trap the free sulfides. A buffer only comparison was used to determine the amount of spiked sulfide that was retrieved. Liver homogenates were prepared as described in 1.2.6. The reagents were added in the following order; Condition 1 (Na\(_2\)S + Zn \(\rightarrow\) spike), 400uL zinc acetate + NaOH mixture \(\rightarrow\) 25uL Na\(_2\)S standard (0.5mM) \(\rightarrow\) 500uL liver homogenate/buffer, Condition 2 (Na\(_2\)S \(\rightarrow\) spike \(\rightarrow\) Zn), 500uL liver homogenate/buffer \(\rightarrow\) 25uL Na\(_2\)S standard (0.5mM) \(\rightarrow\) 400uL zinc acetate + NaOH mixture. Samples were then processed as per section 1.2.3.

### 2.3.8 Measurement of protein s-sulfhydration

The detection of s-sulfhydrated proteins was based on the method described by Mustafa et al (31). S-sulfhydration was detected using a biotin switch assay, in which free thiols (R-SH) are blocked by a highly specific free sulfhydryl-reactive compound, methyl methanethiosulfonate (MMTS). The free s-sulfhydrated thiols (R-S-SH) are then labeled with N-(6-(biotinamido)hexyl)-3′-(2′-pyridydithio)-
propionamide (biotin-HPDP), a compound that interacts with sulphydryl groups. The reason for the selectivity of MMTS towards free thiols and not s-sulphydrated thiols however, is unclear (31, 343).

Liver lysates were prepared by homogenizing ~50 mg tissue in 1mL HEN buffer (250mM HEPES, 1mM EDTA, 0.1mM Neocuproine, 0.1mM Deferoxamine, 1x protease inhibitors) using a bead beater. Homogenates were spun at 20,000 g for 30 mins at 4 °C and the clear supernatant was used for the assay. Protein concentrations of the clear lysates were measured empirically using a Bradford Assay and averaging the value of multiple dilutions of each sample. All samples were then diluted to a concentration of 0.7 mg/mL with HEN buffer. 356 µL (250 µg of protein) of each lysate was then treated with or without 100 µM Na₂S for 30 min at 37°C followed by precipitation with 1 mL of ice cold acetone at -20°C for 20 mins and centrifugation at 950 g at 4°C for 5 mins. Pellets were resuspended in 396 µL of HEN + 2.5% SDS and blocked with 4µL of MMTS (2mM) for 30 mins on a thermomixer set at 50°C and 1400rpm followed by acetone precipitation as described before. Pellets were rinsed twice with 70% acetone (in HEN buffer) and solubilized in 450 µL of biotin-sulphydryl binding buffer (HEN + 2.5 % SDS, 0.8mM biotin-hpdp. Samples were incubated on a rotator in the dark for 3 h followed by acetone precipitation as described before. Pellets were solubilized in 400 µL HEN +1 % SDS and acetone precipitated once more. Finally the pellet containing biotin labelled proteins were solubilized with 30 µL HEN/10 + 1 % SDS and 90 µL neutralization buffer (20mM HEPES, 100mM NaCl, 1mM EDTA and 0.5 % Triton-X 100).

Samples were run on a 10 % SDS-PAGE gel and transferred onto a PVDF membrane. Blots were then blocked with 5 % BSA made in TBST (0.1 % v/v) for 1 h followed by an overnight incubation in 1:1,000 streptavidin-HRP (R&D Systems, USA) made in blocking buffer. Blots were then washed 4 times for 5 mins each in TBST followed by incubation with a chemiluminescence substrate (Western Lightning Ultra by Perkin Ellmer, Netherlands) for 1 min and visualized on a chemi-doc system (Uvitec, UK). Finally, blots were stained coomassie to assess equal protein loading between wells.

In a separate set of experiments, MMTS was replaced with methylsulfonyl benzothiazole (MSBT) and biotin-hpdp was replaced with a biotin-linked cyanoacetate (CN-biotin) while maintaining similar concentrations respectively. This was to test an alternative method of detecting s-sulphydrated proteins as reported by Zhang et al (344).
2.3.9 Data analysis

Data are presented as means \( \pm \) S.E.M. Statistical analysis was performed using the Graphpad Prism Software version 5.03. Multiple comparisons were made using ANOVA with a Bonferonni post-hoc analysis. A p value of less than 0.05 was considered significant. pK value was determined using a non-linear fit (variable slope).

2.4 Results

2.4.1 Effect of pH on stability of sulfides measured by methylene blue assay

The methods by Levitt et al and Shen et al (36, 37) describe the use of an alkaline pH in the preparation of samples to avoid loss of hydrogen sulfide present in the gaseous form. This observation was replicated here and tested over several pH values to determine the optimal pH that would effectively retain free hydrogen sulfide. It also helped determine if the HS\(^-\) species of free sulfide is readily detected by the methylene blue assay. The data showed that sulfides generated from Na\(_2\)S were more stable when prepared in an alkaline buffer where HS\(^-\) is the predominant species and this was detectable using the current methylene blue assay (Figure 2.3). This would be in agreement with the volatility of the neutral hydrogen sulfide species and the stability of the anionic HS\(^-\) species in solution. The observed sulfide stability at pH 9 and 10 was similar, which follows the predicted percentage of HS\(^-\) to be at 99% at pH 9 and 99.9% at pH 10. It would therefore be beneficial to prepare samples in buffers no less than pH 9 to retain the maximal amount of free sulfides in the stable form of HS\(^-\). There was also a noticeable difference in absorbance at 0 mins. These differences could be attributed to the volatility of predominant sulfide species being the gaseous form (hydrogen sulfide) as the pH decreases with respect to the pKa\(_1\) of sulfide (~7 at 25 °C (6)). This, coupled with the time it took to dissolve the sodium sulfide salt and pipette the replicates into the zinc acetate solution may result in some sulfide loss reflected in Figure 2.3.
Figure 2.3 The effect of pH on the stability of sulfide in solution. Known amounts of Na₂S were dissolved at RT in 50 mM sodium phosphate buffer (pH 6 to 8.5) or 50 mM sodium carbonate buffer (pH 9 and 10) to afford a solution with a calculated sulfide concentration of 100 µM. The solutions were then fixed with zinc acetate at specified time points and sulfide levels determined by the current method. Bars represent mean ± S.E.M. (n=3)

2.4.2 Zinc precipitation method of detecting sulfides

The ZnS precipitation and wash method (modified method) was first postulated by Pomeroy et al (346) and put into practice by Gilboa-Garber (345) as a way of detecting inorganic sulfide in biological solutions. These findings were replicated in the present study and showed that this is a robust method with greater sensitivity in comparison to without zinc precipitation; standard curves constructed using both methods (described in section 2.3.5) showed tight deviations and high degree of correlation (current method in buffer : \( R^2 = 0.9981 \), modified method in buffer : \( R^2 = 0.9979 \) ) (Figure 2.4). There was also a higher degree of sensitivity in the modified assay with a discernible lower limit of 0.13 nmole \( (OD_{670} = 0.0070 \pm 0.0014) \) compared to 0.52 nmole \( (OD_{670} = 0.0051 \pm 0.0003) \) without zinc precipitation (Figure 2.4). In our attempt to use this modified method with hydrogen sulfide spiked tissue homogenates, we found that the ratio of zinc acetate, NaOH and sample was not optimal for the removal of biological material while still retaining the ZnS pellet. We therefore optimized the ratio to 7 parts of zinc acetate (1% w/v) to 1 part of NaOH (1.5 M), and this solution was thoroughly mixed and allowed to stand until a granular precipitate formed. This mixture was then used in a 4:5 ratio with the
supernatant of a tissue homogenate (~40 mg/mL). A standard curve was constructed with mouse liver homogenates spiked with pre-precipitated Na$_2$S using the modified method (described in section 2.3.5) and was able to yield a linear regression value of 0.9920 that was slightly elevated compared to the standard curve constructed with buffer only. This could be attributed to the endogenous sulfide from the tissue itself.

![Graph showing OD670 vs Na$_2$S (nmole)](image)

**Figure 2.4 Analysis of the capacity of the methylene blue and zinc precipitation assay to quantify soluble sulfide levels.** A standard curve of serially diluted Na$_2$S was prepared in 50 mM sodium carbonate buffer pH 9, sulfide levels were then determined using either the current assay (▼) or the modified method (■). Additionally, precipitated serially diluted Na$_2$S was spiked into liver homogenate (40 mg/ml) prepared in 50 mM sodium carbonate buffer (pH 9) and detected using the modified method (▲). The inset is a scaled-up representation of the 4 lowest points of the curves. Bars represent mean ± S.E.M. (n=3)

### 2.4.3 Measuring tissue sulfide with the modified method

The pH at which free sulfides are measured within tissue homogenates is dependent on the buffer the tissues are homogenized in. We predicted that homogenates prepared at pH 6 would contain free sulfides predominantly as the neutral species (hydrogen sulfide), and that these species should readily
volatalize during the process of sample preparation and therefore not be detected by the assay. However homogenates prepared at pH 9 should contain free sulfides predominantly as the anionic species, and these should remain in solution, allowing their detection. To confirm this pH dependency in free tissue sulfides, a titration was used to measure sulfides present in liver homogenates as a function of pH (Figure 2.5). The results showed a curve that closely represented the expected dissociation of hydrogen sulfide with a measured pK of about 7.01 (pKa1 of hydrogen sulfide at 25°C ~ 7, (6)). Interestingly, there were reasonably detectable levels of tissue hydrogen sulfide at pH 6, this could be due to the release of bound sulfide under strong alkaline conditions upon addition of the zinc acetate and NaOH solution to the tissue homogenate (will be discussed further in section 2.4.5).

![Graph showing pH vs. H2S (nmollg tissue)](image)

**Figure 2.5 Estimation of sulfide in liver homogenate over different pH. solution.** Sulfide content was measured in mouse liver homogenates (40mg/ml) prepared in 50mM sodium phosphate buffer pH 6, 6.5, 7, 7.5, 8, 8.5 and 50mM sodium carbonate buffer pH 9 using the modified method. The curve is concordant with the dissociation of hydrogen sulfide with a measured pK value of 7.01. Based on these findings, we assumed the difference between sulfides measured at pH 9 and pH 6 as the estimated free sulfides present in the tissue. Bars represent mean ± S.E.M. (n=3)

Based on these observations we proposed that this modified method is able to quantify free tissue hydrogen sulfide based upon the differential detection over the pH range (schematic of method shown in Figure 2.6). As the assay conducted at pH 9 measured both free and bound sulfide, while the assay
conducted at pH 6 only measured background/bound sulfide, the amount of free tissue sulfides could be determined by deducting the measured sulfides in homogenates at pH 6 from pH 9. Using this approach we analyzed a range of tissues and found appreciable amounts of free sulfide in the: lung, liver, kidney and pancreas of BALB/c mice (Figure 2.7). The kidney had the highest amount of 0.119 ± 0.011 nmole per mg of tissue weight, while the lung had the lowest amount at 0.013 ± 0.002 nmole per mg of tissue weight.

Figure 2.6 Schematic of proposed modified method of measuring free tissue sulfide. This figure provides an overview of the modified method. Briefly, aliquots from a piece of tissue were obtained (~50 mg) and homogenised in a sodium phosphate and sodium carbonate buffer of pH 6 and 9 respectively. The supernatant of each homogenate were then treated the same way by undergoing an alkaline-zinc precipitation step of free sulfide followed by pelleting and washing of the trapped ZnS precipitates. The amount of sulfide present in the ZnS precipitates was then detected using the Methylene Blue assay and corrected for protein content in the original homogenate. Free sulfide was determined by deducting the amount of sulfide measured at pH 6 (bound sulfide) from that of pH 9 (total sulfide).
2.4.4 Free sulfide scavenging in a biological solution

This experiment was aimed at determining the retrievability of free sulfide spiked directly into a biological solution. To do so, 12.5 nmole of sulfide was either precipitated with zinc before or after mixing with liver homogenate or buffer only and measured directly using the modified method. The control group of sulfide mixed with buffer only showed a relatively consistent measurement of 12.16 ±0.82 and 11.95±0.20 nmole with or without prior precipitation (Figure 2.8). The liver homogenate group showed a slightly higher sulfide measurement when the spiked sulfide was precipitated before mixing together (13.30±0.37 nmole), this could be due to endogenous free tissue sulfide. However this value was more than halved when the spiked sulfide was mixed directly with liver homogenate and then precipitated with zinc (5.03±0.17 nmole) (Figure 2.8). This suggests that the liver homogenate is able to scavenge substantial amounts of spiked free sulfide; this would have implications on the actual amount of endogenous sulfide that is produced compared to what is detected.
2.4.5 Protein s-sulfhydration

Attempts were made to utilise the biotin-switch assay described by Mustafa et al (31) and Zhang et al (344) to detect s-sulfhydrated proteins. The first method employs an alkylating agent MMTS to differentiate thiols and persulfides. Thiols (-SH) in proteins are first blocked by MMTS. Persulfides (-S-SH) are believed to remain unreacted and be available for subsequent conjugation to biotin-HPDP (31). However, the underlying mechanism of selectivity of MMTS for thiol versus persulfide is unclear and studies have demonstrated that persulfides and thiols should have similar reactivity towards electrophiles such as MMTS (343). This issue was addressed in the second method by the tag-switch protocol. First, a SH-blocking reagent, MSBT, was introduced which should tag both -SH and –S-SH to form an R-S-BT or R-S-S-BT adduct. Next, a biotin conjugated nucleophile, CN-biotin, was used to selectively label the persulfide adducts (R-S-S-BT) as thiol adducts from the first step are thioethers, which are not expected to react with the nucleophile (344).

However I was unable to replicate previously published findings (31, 344) using a positive control suggested in these studies (31, 344). Liver lysates from wild type (WT) C57BL6 mice were treated with or without 100 μM Na₂S for 30 mins at 37 °C. In a third sample, lysates were not treated with Na₂S as well as the biotin labelling compound in order to distinguish background signals arising from
endogenous biotinylated proteins. Equal amount of samples were then resolved on an SDS-PAGE gel, blotted and probed for biotin using a streptavidin-HRP conjugate and ECL detection. There was no detectable difference in staining intensity or pattern between the untreated and Na₂S treated samples using both methods (Figure 2.9) as previously reported (31, 344). Attempts were made to optimize the methods by varying concentration of protein, blocking reagent and detergent to determine if there was non-specific labelling of proteins resulting in high background signal thus masking the potential true difference in the s-sulfhydration state (Appendix 8.4). However there was no observable effect from varying these parameters.

Therefore the initial aim of investigating changes in protein s-sulfhydration state in response to CSE dysregulation was abandoned. There was however an interesting finding, s-sulfhydrated proteins from liver lysates of CSE KO mice showed different banding pattern compared to the WT mice (Figure 2.10). Liver lysates from WT mice showed more signal than the CSE KO with proteins of less than 75 kD in the ‘-bio’ lanes, suggesting higher levels of endogenous biotinylated protein present in the WT liver. This would suggest a novel role for CSE in the synthesis or assembly of endogenously biotinylated proteins.

Figure 2.9 Detecting s-sulfhydrated proteins using biotin-switch and tag-switch assays Liver lysates of wild type mice were assayed for s-sulfhydrated proteins using methods described by Mustafa et al 2009 (A) and Zhang et al 2014 (B). The left panels (biotin blot) represent western blots of biotin labelled s-sulfhydrated proteins probed with streptavidin-HRP and visualised using ECL. The right panels (coomasie stain) are coomasie staining of the same blots to assess equal loading between samples. Unt = untreated lysate, Na₂S = 100µM treated lysate, -bio = untreated lysate that wasn’t incubated with biotin labelling compound. These blots are representative of 3 experiments.
and possibly turnover and transport of biotin as well. Another consequence of this finding is that changes in endogenously biotinylated proteins could affect the interpretation of this assay and a non-biotin labelled group should be included to account for this.

Figure 2.10 Comparing s-sulfhydrated protein profile in liver lysates of WT and CSE KO mice. Liver lysates of WT and CSE KO mice were assayed for s-sulfhydrated proteins using methods described by Zhang et al 2014. The figure represents a western blot of biotin labelled s-sulfhydrated proteins probed with streptavidin-HRP and visualised using ECL. Unt = untreated lysate, Na₂S = 100μM treated lysate, -bio = untreated lysate that is not incubated with biotin labelling compound. This blot is a representative of 3 experiments.

2.5 Discussion

2.5.1 Measuring endogenous free tissue sulfide

Early studies showing endogenous hydrogen sulfide production in mammalian tissue had helped to spark interest in the potential (patho)physiological role(s) of hydrogen sulfide and this has led to a new field of research that is still in its incipient stage. As with any field of study, progress brings about constant re-evaluation of the current body of knowledge. With regards to hydrogen sulfide research, this is most apparent when it comes to the detection and estimation of endogenous levels of free hydrogen sulfide. As mentioned before (section 1.2.3), the issues surrounding the measurement of endogenous free hydrogen sulfide are the (1) passive loss of hydrogen sulfide due to the volatility of the neutral species, (2) high reactivity with a variety of endogenous molecules owing to the reductive
potential of hydrogen sulfide (detailed description in section 1.4.1) and (3) endogenous labile sources of hydrogen sulfide that may be released depending on the assay conditions. These issues have been addressed by Shen et al (36) and Levitt et al (37) in their liquid and gas chromatography based methods. However, as mentioned, the uptake of these methods has been slow despite their specificity and sensitivity. This could be due to the specialised equipment required that is currently not commonplace in a typical laboratory setting and the skills to operate them thus creating an inertia towards adopting these methods.

Therefore, the method described in this chapter was an attempt to refine an existing simple high-throughput method that would fulfil the criteria for specific and sensitive detection of free hydrogen sulfide in tissue with standard laboratory equipment. This method was generally based on the current and widely used spectrophotometric method of detection in the form of the Methylene Blue assay. The major concerns with this assay is the potential release of acid labile sulfur from the homogenate thus resulting in artefactual high measurements and limited sensitivity. The refinements made as a result of this thesis include incorporation of an alkaline-zinc precipitation step at an optimised ratio to separate sulfide from other organic material and preparation of homogenates at dual pH (6 and 9), with the lower pH serving as a reference sample. The combination of these changes should in theory overcome the issue of acid labile sulfide liberation, decrease volatility of free sulfide in solution, increased specificity with introduction of the pH 6 reference sample as well as increased sensitivity with the separation and concentration of sulfide. These changes are elaborated below.

The concept of trapping and precipitating free sulfide with zinc in the current methylene blue method remained appealing and the challenge was to separate the insoluble ZnS precipitate from the homogenate prior to detection to eliminate contaminating sources of labile sulfide. A search through literature yielded a protocol by Gilboa-Garber (1971) that described a method of simultaneously in situ precipitating free sulfide while maintaining the solubility of organic material in the assay media with an alkaline-zinc acetate solution (345). The ZnS precipitates were then pelleted, washed and detected using the methylene blue assay. The intended purpose of this modification by Gilboa-Garber was to remove interfering constituents such as proteins, coloured compounds, sulfur-containing substances and pyrophosphate thus improving the accuracy and sensitivity of the methylene blue assay (345), but the quick and easy separation of insoluble ZnS from the media also offers a simple yet specific method
of detecting free sulfide in tissue homogenates. Another attractive benefit is the flexibility of hydrogen sulfide detection method choice in the resulting ZnS precipitate depending on the instruments available. We therefore chose to use the methylene blue method for its simplicity.

Prior to running the assays, we sought to address the issue of passive hydrogen sulfide loss in the process of preparing tissue homogenates. Total hydrogen sulfide in solution consists of two main species, the neutral hydrogen sulfide and the dissociated anionic HS\(^-\); they exist in equilibria with a pK\(_a\) of ~7.01 at 25 °C (6). The neutral hydrogen sulfide species is gaseous and readily volatilizes out of solution, at pH 7.4 and 25 °C, about 30 % of total free hydrogen sulfide is present as the neutral form thus allowing for substantial passive loss into the atmosphere (347). This relationship is demonstrated in 2.4.1 where there was a clear pH dependent loss of dissolved hydrogen sulfide over time as the pH decreased. At pH 9 (25 °C), 99 % of the hydrogen sulfide species is predicted to exist as the stable HS\(^-\) form in solution and this is supported by the sustained detectable levels of hydrogen sulfide at pH 9 over a period of an hour. Therefore it would be beneficial to prepare tissue homogenates in buffers no less than pH 9 to avoid passive loss of endogenous free hydrogen sulfide; this is in agreement with the Shen and Levitt methods. These results also confirm that the methylene blue method is able to detect the HS\(^-\) species.

Moving forward we were able replicate the Gilboa-Garber method of free hydrogen sulfide detection by alkaline-zinc precipitation and wash followed by a methylene blue assay on the ZnS pellet (section 2.4.2). Standard curves of known Na\(_2\)S concentrations were constructed in buffer and produced a linear correlation of 0.9979. This method was also more sensitive with a discernible lower detection limit of 0.13 nmole (OD\(_{670}\) = 0.0070 ± 0.0014) compared to 0.52 nmole (OD\(_{670}\) = 0.0051 ± 0.0003) using the current methylene blue method for the detection of endogenous free hydrogen sulfide (44, 189). The alkaline-zinc precipitation and wash method was then tested with tissue homogenates with an optimized ratio of NaOH to zinc acetate to ensure complete solubilization of the organic material (section 2.4.3). To test if the method was detecting free hydrogen sulfide, hydrogen sulfide content was measured in liver homogenates over a pH range between 6 and 9 to determine if this relationship reflects the predicted dissociation of hydrogen sulfide at 25°C; assuming this method is able to measure free sulfides, there should be a drop in detectable free sulfide with the reduction of pH due to the volatility and loss of neutral hydrogen sulfide at pH < 9 and this correlation should yield a pK\(_a\)
value close to 7 (6). We were able to demonstrate this relationship using this modified assay with a measured pKₐ of 7.01 (2.4.3). However there was an unexpected detectable signal in homogenates prepared at pH 6, we attributed this to the release of hydrogen sulfide from an alkaline labile source during the alkaline-zinc acetate precipitation step. The most likely source would be sulfur derived from iron-sulfur proteins. This bound sulfur has been shown to be liberated under alkaline-zinc conditions (348, 349). The same studies have also shown cysteinyI residues to be the unlikely source of sulfur release under alkaline-zinc conditions when incubated for short periods. Only a prolonged incubation of 20 h yielded detectable sulfur release from BSA (containing 35 cysteine residues per molecule) (348).

Having shown that the modified method also detected bound sulfur, we then proposed that endogenous free tissue sulfide could be measured by taking the difference between hydrogen sulfide levels in homogenates at pH 9 and pH 6 using the same method. Theoretical calculations predict that >90% of free sulfide exists as the volatile neutral hydrogen sulfide species at pH 6, based on the dissociation constant of sulfide. (6). Therefore, the difference between hydrogen sulfide levels observed in homogenates at pH 9 and pH 6 would represent levels of free tissue sulfide. This, coupled with the physically vigorous sample preparation steps of homogenization and sonication, should ensure that most, if not all, of the free hydrogen sulfide present as the neutral species dissipate into the atmosphere during sample preparation at pH 6. Sample prepared at pH 9 however should have ~99% of the free hydrogen sulfide present as the stable anionic form and therefore retained during the tissue disruption process. Using this approach we have detected appreciable amounts of free hydrogen sulfide in mice kidney, liver, pancreas and lungs, with the kidney having the most and lungs the least (2.4.3). However, these values are several orders more than what had been reported by Levitt et al (2011), ~75 μmol per kg compared to <0.1 μmol per kg in the liver. Since both aliquots of tissue prepared at pH 6 and 9 were subjected to the same alkaline-zinc acetate conditions resulting in a similar final pH value of ~12, the substantial high levels of free hydrogen sulfide detected could be due to anomalously low or high measurement at pH 6 and 9 respectively.

A possible reason for increased free hydrogen sulfide detection at pH 9 is because the reducing activity of thiols is greater in alkaline conditions than at a neutral pH (350). In the presence of major cellular reducing substances such as GSH and cysteine at their physiologic concentrations, hydrogen sulfide has been shown to be released from lysates of cultured neurons and astrocytes at an alkaline pH of...
8.4 (28). However this should also apply to the method by Levitt et al (2011) where tissue samples are homogenized in a pH 9.3 buffer (37) which was not the case. Another possible cause for the high detectable sulfide levels is the reported conversion of disulfide bonds to hydrogen sulfide under strong basic conditions (0.5M NaOH); OH\(^-\) is thought to abstract a proton from the \(\beta\) carbon of the sulfur atom followed by \(\alpha,\beta\) elimination and hydrogen sulfide formation (351, 352). Although a strong alkali-zinc solution was used in the modified protocol described in this chapter, a reference aliquot of tissue sulfide (pH 6) was also carried out in parallel and deducted from the sample aliquot (pH 9) and should therefore cancel out the effect of alkali induced formation of hydrogen sulfide from disulfides.

An interesting observation from the measured free tissue sulfide levels however is the trend, the lungs showed the least while kidney was the highest followed by liver and pancreas. According to a previous report, the kidney has been shown to possess high levels of bound sulfur (sulfur that is release as hydrogen sulfide upon reduction), 324 nmol/g compared to 41 nmol/g in the liver. This disparity would support the argument that the measured free tissue sulfide levels in this thesis could perhaps be derived from bound sulfur instead. The lungs are rich in fibrous tissues that form the scaffold and architecture required for its function, as such about a fifth of the total dry weight is made up of collagen (353) among other connective tissues such as elastin. These structural proteins, collagen and elastin, do not contain sulfur based amino acids and thus make a poorer source of bound sulfur compared to soft tissues such as liver and kidneys.

Although the merits and proof of concept of the “modified method” has been shown in this chapter, the reason for the still substantial disagreement in free tissue sulfide levels detected between this method and the one described by Levitt et al (2011) using gas chromatography (37) is unclear. Further efforts to compare the findings of these two methods would require setting up both methods for a direct side-by-side comparison which would require more time. However, this was not the major objective of this thesis and therefore not pursued.

It is interesting to note that even though the levels of free sulfide reported by Levitt et al (2011) is the lowest detectable levels published thus far in tissues (between 30 and 9200 nmol /kg protein), it has been argued that such levels should still yield tissue concentrations in the high micromolar region and yet there is no detectable hydrogen sulfide odour by the human nose (detection limit ~1 \(\mu\)M) (38). However, the highly reactive nature of hydrogen sulfide should not be overlooked as this may mask
the potential sum of free hydrogen sulfide generated at any one time thus leading to an underestimation of its significance. The results in this thesis have shown that spiked hydrogen sulfide is readily consumed by liver homogenates (2.4.4) and this is in line with previous reports in blood (32), tissue homogenates (33) and cultured cells (53) with half-lives of ~ 130s (32). While hydrogen sulfide catabolism occurs via the mitochondrial sulfide oxidation pathway involving SQR and sulfur dioxygenase enzymes to form sulfite and eventually thiosulfate, it has also been shown that a substantial amount of hydrogen sulfide is also consumed under anaerobic conditions (33). This points to another mode of hydrogen sulfide consumption other than the mitochondrial oxidation pathway, a plausible alternative is consumption via protein s-sulfhydration (refer to section 1.4.2) and this is supported by experiments involving single protein solution of BSA under aerobic and anaerobic conditions with a consumption rate of 1.2 µM min \(^{-1}\) in 5% BSA (32). BSA has one free thiol per molecule. Therefore, while the pursuit in detecting endogenous free hydrogen sulfide is ongoing and rife with issues, it would be worthwhile to also focus on detecting hydrogen sulfide mediated modification of endogenous substances as a marker of endogenous hydrogen sulfide levels owing to its prolific reactivity. This would not provide us with absolute values of hydrogen sulfide levels but may be an indication of instances of increased hydrogen sulfide production/release and potentially lead on to further discovery of its effector mechanisms. At present hydrogen sulfide has been shown to s-sulfhydrate proteins and interact with metal-ion containing proteins apart from its antioxidant ability (section 1.4). Currently, reports on hydrogen sulfide-metal ion interaction have been functional studies and there is not much information on the nature of this interaction and whether it is direct, stable or detectable (107, 133, 160-163). On the other hand, there has been increasing reports of hydrogen sulfide linked endogenous s-sulfhydration (102, 142-146, 167, 354) since it was first reported by Mustafa et al (2009) (31) and the current method of detection is relatively simple. In light of these findings, I attempted to measure s-sulfhydrated proteins as a possible indirect method of determining the endogenous state of hydrogen sulfide production. Results of this effort are described in the next section.
2.5.2 Hydrogen sulfide mediated s-sulfhydration

One of the reported direct hydrogen sulfide mediated effects is the s-sulfhydration modification of free thiols on proteins resulting in formation of protein persulfides and increased cysteine reactivity (31). S-sulfhydration has been suggested to alter protein activity by increasing or inhibiting its activity (31, 141-143) (144) (102, 145, 146). It was my intention to measure endogenous levels of s-sulfhydrated proteins as an indirect indicator of endogenous hydrogen sulfide levels. If successful, I would also be able to compare levels of s-sulfhydrated proteins in an inflammatory response to determine if CSE dysregulation would lead to changes in the protein s-sulfhydration state, presumably through alteration of endogenous hydrogen sulfide synthesis. I elected to use the biotin switch (31) and biotin tag-switch (344) methods to detect and semi-quantitate global levels of s-sulfhydrated proteins by probing for biotin labelled s-sulfhydrated proteins on a western blot, which allows for assessing overall changes in endogenous protein sulfhydration state. The developers of both methods have reported increased protein s-sulfhydration levels in liver lysates following treatment with exogenous hydrogen sulfide (100 μM) (31) (344). I therefore used this published observation as a positive control to validate these methods before proceeding with my actual experimental questions. However I was unable to replicate the findings reported in these studies (31) (344). I had used the laboratory protocol provided by the authors of Mustafa et al 2009 for the biotin switch method and did obtain a signal but there was no increase following treatment of protein lysates with 100 μM hydrogen sulfide (Figure 2.9). Further investigation into the chemistry of the method yielded a surprising find as Pan et al (2013) had reported MMTS to react with both R-SH as well as R-S-SH which should in theory block all thiols and not yield any signal (343). However this background signal was also reported by Pan et al and thought to possibly be due to incomplete thiol blocking or via stepwise thiol-disulfide exchange in a reaction catalyzed by trace free thiols (RSH) (343). The latter involves the reversible nature of MMTS blocking of thiols through reduction of a disulphide bond. Attempts were also made to use an alternative method, a biotin tag-switch method that replaces MMTS with another thiol blocker, MSBT which was reported to bind to both R-SH and R-S-SH groups. The difference with this method is that the subsequent labelling compound, biotin labelled cyanoacetate, would only specifically react with MSBT blocked R-S-SH groups because thiol adducts from the reaction of R-SH
and MSBT are thioethers, which are not expected to react with the nucleophile cyanoacetate. Despite the favourable chemistry, I was unable to detect changes in signal following treatment of tissue lysates with hydrogen sulfide utilising this method (Figure 2.9). Further investigation to try and decrease background signal by increasing MMTS concentration, decreasing protein ratio as well as varying SDS concentration to modulate protein denaturing (to expose free thiols) did not substantially alter or lower the background signal (Figure 8.7). I subsequently tried using another thiol blocker, NEM that has been reported to alkylate both R-SH as well as R-S-SH and is an irreversible method of blocking free thiols, again there was no change in background signal (Figure 8.7). Therefore the evidence points to a limitation in the protocol that prevents complete blocking of all endogenous free thiols (includes both R-SH as well as R-S-SH) which could be high enough to mask differences between untreated and hydrogen sulfide treated lysates.

Despite the outcome, there was an interesting novel finding from this assay which point to a lower level of biotinylated proteins in the livers of CSE KO mice compared to WT. The endogenous biotinylation of proteins is dependent on the availability of biotin (355). Mammals are not able to synthesise biotin and rely on dietary sources as well as endogenous recycling from protein-bound biotin (356). Since the dietary conditions of the WT and CSE KO mice were similar, the observed difference in liver biotinylated proteins between the strains would most likely be due to the capability of endogenous protein-bound biotin recycling, which is mediated by the enzyme biotinidase (355). Biotinidase is able to both hydrolyse and release endogenous biotin and function as a carrier of biotin as well as biotinylate proteins (357). However, it is unclear how CSE deficiency could alter biotinidase activity. A possible mechanism could be an hydrogen sulfide mediated modulation of the redox status of the key cysteine 245 residue in the active site of biotinidase (358). An interesting point to note is that apart from carboxylases, histones have also been shown to be a specific acceptor of biotin transferred from biotinylated biotinidase and thus affect regulation of protein transcription (357).
3 Investigating pancreatic CSE dysregulation in response to acute pancreatitis

3.1 Introduction

CSE is predominantly expressed in peripheral tissues (237) and has been implicated as the key hydrogen sulfide synthesizing enzyme in various models of inflammatory disease (44, 45, 66, 69, 187, 189, 217, 218, 220, 222, 223, 245). Changes in CSE expression levels have been shown in various tissues such as liver (44, 45, 189, 223), kidney (44, 217), lungs (45, 325, 326), gastric mucosa (327), colonic mucosa (328), gall bladder (329) and myocardium (330) in different models of disease and injury. These observed changes in CSE expression levels have been associated with the inflammatory response of the disease or injury and inhibition of endogenous CSE through the use of PAG to either increase or decrease the severity of the disease or injury (359) suggests the importance of CSE regulation with regards to the inflammatory response.

The aetiology of acute pancreatitis is still not completely understood, however it is accepted that the pancreatic acinar cells of the exocrine pancreas plays a central role to the pathogenesis and progression of the ensuing inflammatory response. Currently, premature zymogen and NFκB activation in pancreatic acinar cells are believed to be the two major initiators (254). Uncontrolled premature zymogen activation results in autodigestion of the pancreas thus causing the release of intracellular contents rich in proteases and damage associated molecular pattern molecules (DAMPs) that elicit an inflammatory response. NFκB is a known pro-inflammatory transcription factor that is highly activated at sites of inflammation in diverse diseases (199). Although both zymogen and NFκB activation are observed in pancreatitis, it has been shown that induction of pancreatitis in mice lacking trypsinogen-7, the critical enzyme required for zymogen activation, the disease still progressed without affecting the activation of NFκB (267). This data points strongly to the capacity of pancreatic cells to directly generate pro-inflammatory cues that subsequently elicit a full blown inflammatory response, independent of preceding zymogen activation. Indeed, activated pancreatic acinar cells have been shown to upregulate the expression and secretion of pro-inflammatory cytokines and chemokines. In vitro caerulein stimulation of pancreatic acinar cells has been shown to upregulate expression and secretion of IL-6, IL-1β and MCP-1 via an NFκB mediated pathway (291, 292). Acini isolated from rats with acute pancreatitis also showed substantially elevated levels IL-6, TNF-α, MCP-
1 and MIP-2α via an NFκB mediated pathway (293). These inflammatory mediators are believed to be primarily responsible for the systemic manifestations of acute pancreatitis and its associated distant organ dysfunction (301). Studies using TNF-α and IL-1β receptor knockout mice (306) and MCP-1 blockers (307) have shown substantial protection against pancreatitis and in particular distant organ damage.

CSE is found to be expressed in the pancreas and inhibition of endogenous hydrogen sulfide synthesis by PAG has been shown to decrease chemokine expression (318) and the severity of acute pancreatitis (66). In vitro studies have reported increased CSE expression in pancreatic acinar cells following 1 h caerulein stimulation (304). Pre-treatment of caerulein stimulated pancreatic acinar cells with PAG showed a reduction in secretion of chemokines (318) and expression of adhesion molecules (319). This CSE mediated control of pancreatic acini inflammatory reaction to caerulein stimulation is thought to occur via pathways leading up to NFκB activation; they include substance P-TLR-4 (360) and Src family kinases (SFKs) (319) signalling mechanisms. Though limited, these studies points to a key role of CSE in the inflammatory response associated with acute pancreatitis. It still remains to be shown in vivo if pancreatic CSE expression is upregulated in acute pancreatitis. Additionally there have been no studies that attempt to elucidate the pathway leading up to increased CSE expression in pancreatic acini which could shed light on the pathogenesis of acute pancreatitis.

3.2 Aim

This chapters aims were to determine changes in CSE expression in pancreatic acinar cells and the pancreas under inflammatory conditions associated with pancreatitis as well as the pathway leading up to this. For these studies caerulein stimulated primary pancreatic acini will be used as the in vitro model, and caerulein hyperstimulated mice as an in vivo model of inflammation.
3.3 Materials and methods

3.3.1 Materials

General cell culture reagents and PCR primers were purchased from Life Technologies, USA. LPS purified by phenol extraction was purchased from Sigma-Aldrich, USA. Collagenase type IV was purchased from Worthington-Biochem, USA. Soy derived trypsin inhibitor was purchased from Sigma-Aldrich, USA.

3.3.2 Caerulein induction of acute pancreatitis in mice

Plasma was obtained from mice induced with acute pancreatitis mice using caerulein as described below. Plasma was pooled from individual mice treated with saline or caerulein and frozen in aliquots to make up a batch of working plasma to treat RAW 264.7 cells.

All animal experimentation were approved by the Animal Ethics Committee of the University of Otago (Protocol C57/11) and performed according to the guidelines. Caerulein was obtained from Bachem (Bubendorf, Switzerland) and reconstituted in sterile saline to a final concentration of 1 mg/100 mL and frozen in 1.5 mL aliquots. WT and CSE KO C57BL6 mice (male, 20–25 g) were assigned randomly to control or experimental groups. WT mice were obtained from the Christchurch Animal Research Area. Animals were given hourly intraperitoneal (i.p.) injections of normal saline (0.9% wt/vol NaCl) or caerulein (50 µg/kg) for 10 h, as described previously (305, 361). For pain relief, all mice were given 3 subcutaneous (s.c.) doses of buprenorphine (0.2 mg/kg) per hour before treatment, as well as 3 h and 7 h into the treatment. One hour after the last caerulein/saline injection, animals were sacrificed by an i.p. injection of sodium pentobarbital. Blood samples were drawn from the right ventricle using heparinized syringes and centrifuged (1,000 g for 10 min, 0–4 °C). Thereafter, plasma was aspirated and stored at -80°C for future use.

3.3.3 Primary pancreatic acinar fragment preparation and treatment

The media use for isolating primary pancreatic acinar cells consisted of DMEM supplemented with 0.1% w/v BSA, 0.05% w/v trypsin inhibitor and oxygenated with 95% oxygen and 5% CO₂ before use.
The method of isolation was based on Bruzzone et al (362). Freshly removed pancreases were placed in ice cold media to rinse off excess blood and then transferred to a 1.5mL microfuge tube on ice containing media supplemented with collagenase type IV at a final concentration of ~400 U/mL. Each individual pancreas was minced finely using scissors to pieces ≤1mm in diameter. The minced pancreas was then transferred to a glass vial, topped up with collagenase solution, sealed tightly and in a 37 °C shaking water bath for 10 mins. After that the vial was shaken vigorously by hand for 1 min and placed back into the bath for a further 5 mins after which it was vigorously shaken by hand for a final 30 s. The digest was then briefly triturated with a large orifice plastic pasteur pipette and layered onto a 4% BSA solution in a 15 mL tube. This was then centrifuged at 50 g for 2 mins, the collagenase and BSA layers were discarded and the cells were rinsed with fresh media 2 times by centrifuging at 50g for 70 s each time. The pellet was the triturated in fresh media and the top layer of fine tissue fragments as the bigger clumps settled was transferred to a fresh tube to be used for experiments. These fine fragments consisted of cell clusters of 5 to 20 cells and had viability of more than 90% as determined by trypan blue exclusion.

The final pancreatic fragments were distributed into 1.5 mL microfuge tubes and treated with either saline or caerulein at a final concentration of 100 nM. They were incubated at 37 °C on a rotator for 60 mins after which they were harvested by centrifuging at 1000 g for 5mins and frozen at -80 °C for western blot analysis or processed for RNA extraction as described below.

### 3.3.4 RT-PCR and real-time quantitative PCR (qPCR)

#### 3.3.4.1 RNA extraction

Pellets of pancreatic tissue fragments were analysed for CSE and CBS expression using conventional PCR and qPCR. Total RNA was extracted using the phenol-chloroform method with Trizol purchased from Life Technologies, USA. Pancreatic acinar cells contain large amount of RNases that readily degrade RNA upon lysis of the cells. Therefore pancreatic tissue fragments were first pelleted at the end of the experiment and resuspended in RNA later (Sigma-Aldrich, USA) and kept at 4 °C for 30 mins. Samples were then pelleted at 50 g for 1 min, resuspended in 500µL Trizol and left at RT for 5mins. 100 µL chlorofom was added and tubes were shaken vigorously for 15 s and incubated at RT for 10
mins after which they were centrifuged at 12,000 g for 15 mins at 4 °C. The clear supernatant was then transferred to a fresh 1.5mL microfuge tube. 100 μL isopropanol was added, tubes were mixed gently and incubated at RT for 10mins followed by centrifugation at 12,000 g for 10 mins at 4 °C. The supernatant was discarded, the RNA pellets rinsed with 1 mL chilled 70% ethanol and pelleted again at 7,500 g for 5 mins at 4 °C. The supernatant was then discarded and the tubes air dried at RT. Finally the RNA pellets were reconstituted with an appropriate volume of Milli-Q water and read on a nano drop to determine RNA quantity and quality.

3.3.4.2 cDNA synthesis

cDNA synthesis was carried out with the High-Capacity cDNA Reverse Transcription Kit (Life Technologies, USA) using an MMLV reverse transcriptase enzyme. A master mix of the reaction mixture was prepared according to the manufacturers protocol, this consisted of 2 μL reaction buffer, 0.8 μL dNTP mix (4mM), 1 μL random primers, 1 μL Oligo(dT)20, 1 μL reverse transcriptase enzyme and 4.2 μL Milli-Q water per reaction. Template was prepared by using 1 μg of total RNA and topping up with Milli-Q water to a total of 10 μL. For each sample, 10 μL of the master mix was added to 10 μL of the prepared template for a final reaction of 20 μL in PCR tubes. Reverse transcription was performed on a thermal cycler (Applied Biosystems 2720, USA) using the following conditions; 25°C for 25 mins > 37°C for 120 mins > 85°C for 5 mins > 4°C hold. Samples were then stored at -80 °C for further use.

3.3.4.3 PCR and qPCR

Primers for CSE, CBS, β-actin were designed using the NCBI primer blast software and 18S was based on a publication (363) and purchased from Life Technologies (USA), details are shown in Table 5. PCR and qPCR were carried out with the BIOTAQ PCR kit using a TAQ polymerase enzyme (Bioline, UK). Amplifications were run in 20 μL reaction volumes with 1 μL of cDNA as the template. For conventional PCR, each reaction consisted of 0.5 U of Biotaq DNA polymerase, 1x NH₄ reaction buffer, 2.5 mM MgCl₂, 0.2 mM dNTP, 5 μM of forward primer and 5 μM of reverse primer. For qPCR, a DNA dye Eva Green (Biotium, USA) was added to the reaction mixture described. To determine primer specificity, samples were run on a 1.5 % agarose gel for 35 cycles and visualised on a gel-doc system (Uvitec, UK) to assess
the amplicon size and presence of non-specific bands. qPCR was performed on a Roche Light Cycler 480 II. Comparative levels of CSE expression was quantified using β-actin or 18S as the reference gene and based on calculations described by Pfaffl et al (364).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Annealing temp (°C)</th>
<th>Fragment size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSE</td>
<td>5'-GAATGGGATGGCGCTGCG-3'</td>
<td>5'-GCCCAGACACTGCGGT-3'</td>
<td>60</td>
<td>115</td>
</tr>
<tr>
<td>CBS</td>
<td>5'-GGCTGAAAGTGGCCGCA-3'</td>
<td>5'-CTTGCTGGACATGCCATTGTG-3'</td>
<td>60</td>
<td>214,172*</td>
</tr>
<tr>
<td>β-actin</td>
<td>5'-CTGTCAATGGCAGGCGTCAACCC-3'</td>
<td>5'-ACATGCGGAGCGGTTGTCG-3'</td>
<td>60</td>
<td>128</td>
</tr>
<tr>
<td>18S</td>
<td>5'-GTAACCCGTGAAGCACCATT-3'</td>
<td>5'-CCATCCAATCGGTAGTACG-3'</td>
<td>60</td>
<td>151</td>
</tr>
</tbody>
</table>

Table 5 PCR and qPCR primers and conditions. Shows the primers used to amplify the genes of interest with corresponding annealing temperature. * denotes two amplicons from splice variants.

3.3.5 Western blotting

Pellets of the RAW264.7, pancreatic tissue fragments and pancreatic tissue were analysed for CSE expression by western blot. Total cell lysate was prepared by triturating pellets in ice cold RIPA buffer (50 mM TRIS pH7.5, 150 mM NaCl, 1% v/v NP-40, 0.5% w/v sodium deoxycholate, 0.1% w/v sodium dodecylsulfate supplemented with protease inhibitors (HALT by Pierce Biotechnology, USA)) and incubating on ice and vortexed vigorously periodically. Pancreatic tissue lysate was prepared by homogenising ~50 mg tissue in ice cold RIPA buffer supplemented with protease inhibitors with a Labserv homogeniser and incubated on ice. After 30 mins, samples were centrifuged at 30,000 g for 30 mins at 4 °C. The clear supernatant (total cell or tissue lysate) was collected and measured for protein content using the Bio-rad DC Protein Assay (Bio-rad, USA) and BSA protein standards as a reference. Samples were run on a 10 % w/v SDS-PAGE gel under reducing conditions with equal loading of 20 μg of protein of each sample per well. Gels were run at a constant 200 V until the gel front had run out and then transferred onto a 0.45 μm nitrocellulose membrane (Whatman, USA) at a constant 100 V for 1 h using a Bio-rad system. Running buffer consisted of 25 mM TRIS, 192 mM Glycine and 0.1 % w/v SDS while transfer buffer consisted of 25 mM TRIS and 192 mM Glycine. Blots were then rinsed in TBS supplemented with 0.05% Tween-20 (TBST) and blocked with 5% non fat dry milk made in TBST. Blots were cut at the 37 kDa Mw marking and each portion of the membrane was probed for either
CSE (~42 kDa) or GAPDH (~35 kDa) and HPRT (~23 kDa) at 1:1000 dilutions in blocking buffer at 4 °C overnight. Blots were then washed 4 times with TBST for 5 mins each and probed with the corresponding HRP conjugated secondary antibody (1:20,000) for 2 h at RT. Blots were then washed 4 times with TBST for 5 mins each with a final TBS rinse for 5 mins. Blots were then incubated with a chemiluminescence substrate (Western Lightning Ultra by Perkin Ellmer, Netherlands) for 1 min and visualized on a chemi-doc system (Uvitec, UK). Bands were semi quantitated using the Alliance 4.2 software and compared for relative intensities. Primary mouse anti CSE antibody was from Abnova (Taiwan), primary rabbit anti GAPDH and HPRT antibodies were from Santa Cruz Biotechnology (USA), secondary goat anti mouse HRP conjugated antibody was from Pierce Biotechnology (USA) and secondary goat anti rabbit HRP conjugated antibody was from Santa Cruz Biotechnology (USA).

3.3.6 Immunohistochemistry of pancreatic CSE expression.

The induction of pancreatitis is described in section 3.3.2. Pancreases from control mice and mice with acute pancreatitis were then harvested and stained for CSE expression. Upon harvest, pancreases were fixed in 4% buffered paraformaldehyde and sent to the Christchurch Hospital Histopathology Department to be processed and set into paraffin blocks. 4 μm thick formalin fixed paraffin embedded tissues sections were cut using a Leica microtome and immobilized onto superfrost coated glass slides (Menzel-Glaser, Germany). Each slide had a pair of pancreatic sections from wild type saline and caerulein treated mice. Slides were left at RT to air dry for at least a week before use. Staining conditions were optimized using pancreatic sections from mice lacking the CSE gene, results are shown in appendix 8.3.

Staining was performed with the Rabbit specific HRP/DAB (ABC) Detection IHC Kit (Abcam, Cambridge UK). Sections were rehydrated through a xylene-alcohol series (Xylene 1 > xylene 2 > Xylene+ethanol (1:1) > 95% ethanol > 70% ethanol > 50% ethanol > water) by immersing slides in each reagent for 5mins. This was followed by an antigen retrieval step by incubating slides in a 10 mM Sodium citrate, 0.05% Tween 20, pH 6.0 solution at 97 °C for 20 mins in a water bath. Slides in buffer were removed and allowed to cool to room temperature followed by immunostaining as described in the manufacturers’ protocol. Sections were blocked for 30 mins at RT with block buffer supplemented with
1% w/v BSA followed by an overnight incubation at 4°C with primary antibody; rabbit anti- CSE (Abcam, UK) at 1:1,000 dilution in TBS with 5% w/v BSA. This was followed by a hydrogen peroxide block for 15 mins, secondary antibody incubation for 60 mins, streptavidin incubation for 20 mins and DAB substrate incubation for 30 s immediately followed by a rinse and soak in water. TBST (0.025% w/v) was used as the wash buffer between each step and rinsed with TBS after each wash cycle. Sections were then counterstained with hematoxylin, dehydrated through a xylene-alcohol series described above and mounted. Five fields of view (FOV) taken from the centre and four ends of the section in a north, east, south, west manner were taken from each section with a 20x objective lens on a Zeiss Axioscope.Z1 microscope. CSE staining intensity was measured using the Zeiss Axiovision 4.6.3 software by first setting the colour selection and threshold to selectively analyse the DAB stained portions and excluded non-specific stained portions such as the nuclei and intensely stained artefacts. This allowed automated detection of positive DAB staining within each field of view. The software then generates a report consisting of individual sections that were deemed positively stained along with the percentage of area it occupies within the field of view and the corresponding staining intensity. Only areas that occupied > 0.1 % of the area within a field of view were considered to exclude artifacts and stained individual cells (monocytes and neutrophils). Sections of saline and caerulein treated pancreases within one slide were compared in a pair-wise manner; this is to avoid possible errors that may arise from differences in staining between separate slides.

3.3.7  Pancreatic hydrogen sulfide synthesizing activity

hydrogen sulfide synthesizing activity in pancreatic homogenates was measured with a modified protocol based on methods described previously (365). ~50 mg pancreatic tissue was homogenised in 50 mM ice-cold potassium phosphate buffer (pH 7.4) using a Labserv homogeniser. The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.4), L-cysteine (20 μl, 10 mM), pyridoxal 5’-phosphate (20 μl, 2 mM), saline (30 μl), and 11% w/v tissue homogenate (430 μl). The reaction was performed in tightly parafilm-sealed microfuge tubes (with lids taken off) and initiated by transferring the tubes from ice to a water bath at 37°C. After incubation for 30 min, 1% w/v zinc acetate (250 μl) was injected in to trap evolved hydrogen sulfide followed by 10% v/v trichloroacetic acid (250 μl) to denature the protein and stop the reaction. Subsequently, N,N-dimethyl-p-phenylenediamine sulf
(20 μM, 133 μl) in 7.2 M HCl was added, immediately followed by FeCl₃ (30 μM, 133 μl) in 1.2 M HCl. Samples were left to incubate at room temperature in the dark for 20 min and subsequently pelleted. The absorbance of the clear supernatant was measured with a 96-well microplate spectrophotometer at 670 nm. The hydrogen sulfide concentration was calculated against a calibration curve of Na₂S. Results were then corrected for the protein content of the tissue sample determined by the Bio-rad DC protein assay and are expressed as nmole hydrogen sulfide formed/mg protein.

3.3.8 Statistical analysis

Data are presented as means ± S.E.M. Statistical analysis was performed using the Graphpad Prism Software version 5.03. Multiple comparisons were made using ANOVA with a Bonferoni post-hoc analysis. Comparisons of two data sets were made with an unpaired Student’s t-test except for the analysis of in vivo pancreatic CSE expression using IHC which was compared using a paired Student’s t-test. A p value of less than 0.05 was considered significant.

3.4 Results

In vivo pancreatitis experiments have so far shown increased pancreatic hydrogen sulfide synthesizing activity following induction and this was abrogated with the use of PAG, a CSE inhibitor (66, 318). This suggests a possible upregulation of pancreatic CSE and its relevance to the progression of the disease, however there has not been direct evidence of CSE upregulation in pancreatitis at the time this thesis was conceived and carried out, apart from one in vitro study that showed increased CSE expression in caerulein stimulated pancreatic acinar cells (304). The following experiments were carried out to determine if CSE is upregulated in the exocrine portion of the pancreas in vivo during acute pancreatitis by western blotting and immunohistochemistry. qPCR was not used due to the difficulty in obtaining good quality RNA from pancreatic tissue.

3.4.1 Pancreatic CSE expression and activity in acute pancreatitis

Pancreatic CSE expression and hydrogen sulfide synthesizing activity was measured in an in vivo model of caerulein induced acute pancreatitis in mice. Western blot analysis showed a significant ~33% ± 8
% increase in pancreatic CSE expression in pancreatitic mice as compared to the saline treated control mice (p<0.05) (Figure 3.1a and b). There was a concomitant significant increase in pancreatic hydrogen sulfide synthesizing activity at a similar magnitude (~41 ± 14 %) (p<0.05) (Figure 3.1c).

Figure 3.1 Pancreatic CSE expression and hydrogen sulfide synthesizing activity in acute pancreatitis. Pancreatic CSE expression was measured by western blot following a 10 h induction of acute pancreatitis in mice. Hydrogen sulfide synthesizing activity was also measured using the methylene blue method. There was a significant increase in CSE expression as shown in the representative blot (A) and densitometry analysis (B). This is supported by a detectable increase in pancreatic hydrogen sulfide synthesizing activity (C). Bars represent mean ± S.E.M. (n=5).* denotes significant difference over saline control (p<0.05)

Immunohistochemical staining of CSE was used to visualise and confirm this observation. Staining of pancreatic sections showed a significant increase in CSE expression in the pancreatitic mice compared to the saline controls (~1.11 ± 0.03 fold increase) (p<0.05) and was most evident in the exocrine portion of the pancreas comprising of acinar cells. Figure 3.2a represents the CSE stained pancreas of mice treated with saline and Figure 3.2b is that of mice treated with caerulein.
Figure 3.2 Pancreatic immunohistochemical staining of CSE in acute pancreatitis. Formalin fixed paraffin embedded (FFPE) sections of control and pancreatitic mice were immunostained for CSE and detected by DAB yielding a brown stain in areas of positive detection. The cell nuclei in these sections were then counterstained with haematoxylin (purple stain). Representative sections show an observable increase in staining intensity of CSE in the pancreas of mice treated with caerulein (B) compared to the saline control (A). A densitometric analysis using the Axiovision software shows a significant increase in staining density (C). Bars represent mean ± S.E.M. (n=5). *denotes significant difference (p<0.05). Endocrine portion of the pancreas (shown by arrow) was weakly stained in comparison to the exocrine and there was no difference in response to pancreatitis (D left = Control, D right = pancreatitis)

3.4.2 CSE & CBS expression in pancreatic acinar fragments in response to caerulein stimulation

Pancreatic acinar cells form the exocrine portion of the pancreas and are thought to play a key role in the inflammatory response associated with pancreatitis. The in vitro stimulation of primary isolated
pancreatic acinar fragments with caerulein mimics the in vivo model of pancreatitis and is used to study the pathogenesis and pathophysiology of pancreatitis (366). Here I sought to determine if caerulein stimulated pancreatic acinar fragments from mice result in altered CSE and CBS expression using qPCR and western blotting as it was possible to obtain good quality RNA from these fresh isolates. Figure 3.3 shows no significant change in CSE and CBS expression between caerulein and saline treatment after 1 h.

![Figure 3.3 CSE & CBS expression in pancreatic acinar fragments in response to caerulein stimulation.](image)

qPCR (A) & western blot (B) of CSE and qPCR of CBS (C) in pancreatic acinar cells following 1 h caerulein stimulation. There was no significant change in CSE and CBS expression in the caerulein stimulated group over saline control. Bars represent mean ± S.E.M. (n=3).

### 3.5 Discussion

This chapter confirms increased pancreatic acini CSE expression in response to acute pancreatitis with direct evidence using western blotting and immunohistochemistry to quantify as well as determine the location. The contribution of CSE expression in pancreatitis will be the subject of further study in chapter 6.

#### 3.5.1 Pancreatic CSE upregulation following acute pancreatitis

Both CSE and CBS are expressed in the pancreas; CBS is present in the exocrine as well as the endocrine portion of the pancreas while CSE was only detectable in the exocrine portion (17). The exocrine portion of the pancreas is mainly made up of pancreatic acinar cells that are responsible for the synthesis of digestive enzymes, these cells are also thought to play an active role in the pathogenesis
of pancreatitis and propagation of the associated inflammatory response (267). Increased pancreatic hydrogen sulfide synthesizing activity has been reported in pancreatitis and was inhibited through the use of PAG resulting in an amelioration of the disease (66, 244, 318). This suggests that pancreatic CSE expression is upregulated in pancreatitis and plays a key pro-inflammatory role in the disease, there was however very little direct evidence of this upregulated response except for an in vitro report of caerulein stimulated pancreatic acinar cells (304). Here, I have shown that pancreatic CSE expression is upregulated by western blotting following caerulein induced acute pancreatitis in mice and this is supported by immunohistochemistry that shows increased staining in the exocrine portion of the pancreas (Figure 3.1 & Figure 3.2). These results are supported by a recent publication that used a sodium taurocholate model of pancreatitis in rats (69). Taken together, these findings affirm the upregulation of pancreatic CSE in response to pancreatitis and this occurs in the pancreatic acinar cells.

### 3.5.2 CSE expression in caerulein stimulated pancreatic acini

Following confirmation that CSE is upregulated in the pancreas during pancreatitis, the next step was to investigate the molecular mechanisms leading up to this increased CSE expression using an in vitro system. Primary pancreatic acinar fragments were prepared according to an established protocol (362) and hyperstimulated with caerulein for 1 h to induce a pancreatitis like state. In contrast to the in vivo findings, there was no detectable change in CSE expression by qPCR and western blotting. This discrepancy could be due to limitations of the in vitro system that does not include the complex interaction of pancreatic acinar cells with other components of the inflammatory response such as resident tissue macrophages and circulating neutrophils. Another factor could be due to the shorter induction period used in the in vitro system. However it is interesting to note that there has been one study that reported caerulein mediated upregulation in pancreatic acinar CSE expression using standard PCR (304). The protocols of isolation and stimulation used in that study and this thesis are essentially the same with the exception that the study resuspended pancreatic acini in media containing epidermal growth factor (EGF). EGF is used to support long term culture of pancreatic acini for up to 5 days (367) and has been shown to activate MAPKs in a pathway distinct from caerulein (368). The use of EGF could possibly have a synergistic or alternate activation effect on pancreatic acini stimulated with caerulein resulting in the observed CSE dysregulation.
4 Investigating macrophage CSE dysregulation in response to acute pancreatitis

4.1 Introduction

The inflammatory response comprises a complex interplay between inflammatory cues/stimuli such as cytokines, chemokines and eicosanoids with immune effector cells that include granulocytes (innate response) and lymphocytes (adaptive response). Macrophages represent a major component of the innate response system consisting of closely related cells of bone marrow origin, including blood monocytes, and tissue macrophages. Macrophages play a critical role in the initiation, maintenance, and resolution of inflammation through antigen presentation, phagocytosis, and immunomodulation by producing various cytokines and growth factors (313). In pancreatitis, macrophages are thought to play a key role in the initiation and propagation of the inflammatory response (297). Resident peritoneal macrophages have been implicated as the initial target due to their proximity to the pancreas and exposure to the ascitic fluid generated during pancreatitis. Studies have shown the activation of peritoneal macrophages in response to pancreatitis and to in vitro exposure with pancreatitis associated ascitic fluid (PAAF) (300, 310-312, 369, 370). This activation was found to be intense and occur in the early stages of pancreatitis (370) causing a massive release of pro-inflammatory mediators into the peritoneal ascitic fluid (369) that in turn propagates the inflammatory response.

There have been limited studies on the effect of CSE expression and endogenous hydrogen sulfide synthesis in macrophages, 6 in total. The first study by Oh et al (201) showed increased CSE expression following 4 h of LPS (1 μM) stimulation in RAW 264.7 cells. The expression of CSE was associated with a decrease in iNOS synthesis and NO production; this was supported by a subsequent study that also measured increased hydrogen sulfide synthesis in LPS stimulated RAW 264.7 cells using a hydrogen sulfide specific electrode (55). There have been a couple of studies investigating ox-LDL stimulated macrophages; these studies have shown basal CSE & CBS/endogenous hydrogen sulfide to play a role in suppression of NFκB activation through sulfhydration of the cysteine-38 residue of the p65 subunit and ox-LDL stimulation was found to decrease CSE & CBS expression/endogenous hydrogen sulfide leading to activation of these macrophages (205, 321). Conversely, there have been reports on the role of CSE expression as a pro-inflammatory mediator in macrophages; CSE was shown to be a positive
regulator of TNF-α, IL-1β, IL-6 and MCP-1 release through activation of NFκB in response to LPS stimulation (249, 250).

These initial studies provide an insight to the possible role of CSE and hydrogen sulfide in the regulation of macrophage function. However with the exception of LPS and ox-LDL stimulation of macrophages, the role of macrophage CSE expression has not been investigated in any inflammatory disease. Additionally there have been no studies that attempt to elucidate the pathway leading up to increased CSE expression in macrophages which could shed light on the upstream regulator of hydrogen sulfide synthesis in macrophages; a key effector cell of the innate immune response.

Studies on the effect of exogenous hydrogen sulfide on macrophages in vitro have employed hydrogen sulfide concentrations between 50 to 100 μM (55, 201, 203, 205, 321). All of these studies have shown that exogenous hydrogen sulfide dampens the effect of LPS and ox-LDL activated macrophages by decreasing the nitric oxide, cytokine and cell adhesion molecule production. However, these hydrogen sulfide concentrations are multiple orders higher than the levels currently detectable in tissue and the circulation (discussed in section 2.4.5) thus putting its physiological relevance to question. If so, what is the role of CSE/endogenous hydrogen sulfide in macrophages in inflammation? There are currently a handful of studies that attempt to address this question. Inflammatory stimuli have been shown to alter CSE expression levels in primary and macrophage cell lines; LPS stimulation caused an increase (55, 201, 249) while ox-LDL elicited a decrease (321) in CSE expression. Using gene knockdown and over expression models, this dysregulation of CSE expression was reported to have both a pro and anti-inflammatory effect in stimulated macrophages; increased CSE expression decreased NO production (55) and TNF-α release (321) in response to LPS and ox-LDL stimulation respectively but was also associated with increased cytokine release following LPS stimulation (249). In this chapter, I investigated macrophage CSE expression levels in response to LPS and acute pancreatitis as well as the molecular mechanisms involved.

4.2 Aim

This chapters aims were to determine changes in CSE expression in macrophages under inflammatory conditions associated with pancreatitis as well as the pathway leading up to this. For these studies,
RAW264.7 mouse macrophage cell line were used and stimulated with LPS as a classic activator for optimisation purposes. Finally these cells will be stimulated with plasma from pancreatitic mice in an attempt to replicate the *in vivo* activation of macrophages during pancreatitis.

### 4.3 Materials and methods

#### 4.3.1 Materials

General cell culture reagents and PCR primers were purchased from Life Technologies, USA. LPS purified by phenol extraction was purchased from Sigma-Aldrich, USA. The sp1 inhibitor, Mithramycin A was purchased from Tocris Bioscience, UK. The Akt inhibitor, Triciribine and PI3K inhibitor, LY294002 were purchased from Sigma, USA. Caerulein was obtained from Bachem, Switzerland.

#### 4.3.2 RAW264.7 culture and experiments

The mouse macrophage cell line was maintained in DMEM supplemented with 10% FBS and antibiotics. For the LPS stimulation experiments, cells were seeded at 50x10⁴ cells/cm² and the media was refreshed following day with or without 1 μg/mL LPS. Cells were harvested 24 h later with cell scrapers and rinsed once with PBS before freezing the pellets at -80°C for further analysis. In experiments involving inhibitors, cells were treated with the inhibitors 1 h prior as well as throughout the course of LPS stimulation at the concentrations mentioned. For the plasma stimulation experiments, the cells were treated with DMEM supplemented with 10% plasma from either saline or caerulein treated mice and harvested 24 h later. In some experiments, the harvested cells were divided to be analysed by qPCR and western blotting.
4.3.3 **RT-PCR and real-time quantitative PCR (qPCR)**

4.3.3.1 **RNA extraction**

Pellets of RAW264.7 were analysed for CSE and CBS expression using conventional PCR and qPCR. Total RNA was extracted using the phenol-chloroform method with Trizol purchased from Life Technologies, USA. Frozen RAW 264.7 cell pellets were triturated in 500μL Trizol and left at RT for 5mins. 100 μL chlorofom was added and tubes were shaken vigorously for 15 s and incubated at RT for 10 mins after which they were centrifuged at 12,000 g for 15 mins at 4 °C. The clear supernatant was then transferred to a fresh 1.5mL microfuge tube. 100 μL isopropanol was added, tubes were mixed gently and incubated at RT for 10mins followed by centrifugation at 12,000 g for 10 mins at 4 °C. The supernatant was discarded, the RNA pellets rinsed with 1 mL chilled 70% ethanol and pelleted again at 7,500 g for 5 mins at 4 °C. The supernatant was then discarded and the tubes air dried at RT. Finally the RNA pellets were reconstituted with an appropriate volume of Milli-Q water and read on a nano drop to determine RNA quantity and quality.

4.3.3.2 **cDNA synthesis**

cDNA synthesis was carried out with the High-Capacity cDNA Reverse Transcription Kit (Life Technologies, USA) using an MMLV reverse transcriptase enzyme. A master mix of the reaction mixture was prepared according to the manufacturers protocol, this consisted of 2 μL reaction buffer, 0.8 μL dNTP mix (4mM), 1 μL random primers, 1 μL Oligo(dT)20, 1 μL reverse transcriptase enzyme and 4.2 μL Milli-Q water per reaction. Template was prepared by using 1 μg of total RNA and topping up with Milli-Q water to a total of 10 μL. For each sample, 10 μL of the master mix was added to 10 μL of the prepared template for a final reaction of 20 μL in PCR tubes. Reverse transcription was performed on a thermal cycler (Applied Biosystems 2720, USA) using the following conditions; 25°C for 25 mins > 37°C for 120 mins > 85°C for 5 mins > 4°C hold. Samples were then stored at -80 °C for further use.
4.3.3.3 PCR and qPCR

Primers for CSE, CBS, β-actin were designed using the NCBI primer blast software and 18S was based on a publication (363) and purchased from Life Technologies (USA), details are shown in Table 5. PCR and qPCR were carried out with the BIOTAQ PCR kit using a TAQ polymerase enzyme (Bioline, UK). Amplifications were run in 20 µL reaction volumes with 1 µL of cDNA as the template. For conventional PCR, each reaction consisted of 0.5 U of Biotaq DNA polymerase, 1x NH_4 reaction buffer, 2.5 mM MgCl_2, 0.2 mM dNTP, 5 µM of forward primer and 5 µM of reverse primer. For qPCR, a DNA dye Eva Green (Biotium, USA) was added to the reaction mixture described. To determine primer specificity, samples were run on a 1.5 % agarose gel for 35 cycles and visualised on a gel-doc system (Uvitec, UK) to assess the amplicon size and presence of non-specific bands. qPCR was performed on a Roche Light Cycler 480 II. Comparative levels of CSE expression was quantified using β-actin or 18S as the reference gene and based on calculations described by Pfaffl et al (364).

4.3.4 Western blotting

Pellets of RAW264.7 cells were analysed for CSE expression by western blot. Total cell lysate was prepared by triturating pellets in ice cold RIPA buffer (50 mM TRIS pH7.5, 150 mM NaCl, 1% v/v NP-40, 0.5% w/v sodium deoxycholate, 0.1% w/v sodium dodecylsulfate supplemented with protease inhibitors (HALT by Pierce Biotechnology, USA)) and incubating on ice and vortexed vigorously periodically. Pancreatic tissue lysate was prepared by homogenising ~50 mg tissue in ice cold RIPA buffer supplemented with protease inhibitors with a Labserv homogeniser and incubated on ice. After 30 mins, samples were centrifuged at 30,000 g for 30 mins at 4 °C. The clear supernatant (total cell or tissue lysate) was collected and measured for protein content using the Bio- rad DC Protein Assay (Bio- rad, USA) and BSA protein standards as a reference. Samples were run on a 10 % w/v SDS-PAGE gel under reducing conditions with equal loading of 20 µg of protein of each sample per well. Gels were run at a constant 200 V until the gel front had run out and then transferred onto a 0.45 µm nitrocellulose membrane (Whatman, USA) at a constant 100 V for 1 h using a Bio- rad system. Running buffer consisted of 25 mM TRIS, 192 mM Glycine and 0.1 % w/v SDS while transfer buffer consisted of 25 mM TRIS and 192 mM Glycine. Blots were then rinsed in TBS supplemented with 0.05% Tween-20
(TBST) and blocked with 5% non fat dry milk made in TBST. Blots were cut at the 37 kDa Mw marking and each portion of the membrane was probed for either CSE (~42 kDa) or GAPDH (~35 kDa) and HPRT (~23 kDa) at 1:1000 dilutions in blocking buffer at 4 °C overnight. Blots were then washed 4 times with TBST for 5 mins each and probed with the corresponding HRP conjugated secondary antibody (1:20,000) for 2 h at RT. Blots were then washed 4 times with TBST for 5 mins each with a final TBS rinse for 5 mins. Blots were then incubated with a chemiluminescence substrate (Western Lightning Ultra by Perkin Ellmer, Netherlands) for 1 min and visualized on a chemi-doc system (Uvitec, UK). Bands were semi quantitated using the Alliance 4.2 software and compared for relative intensities. Primary mouse anti CSE antibody was from Abnova (Taiwan), primary rabbit anti GAPDH and HPRT antibodies were from Santa Cruz Biotechnology (USA), secondary goat anti mouse HRP conjugated antibody was from Pierce Biotechnology (USA) and secondary goat anti rabbit HRP conjugated antibody was from Santa Cruz Biotechnology (USA).

4.3.5 Statistical analysis

Data are presented as means ± S.E.M. Statistical analysis was performed using the Graphpad Prism Software version 5.03. Multiple comparisons were made using ANOVA with a Bonferoni post-hoc analysis.

4.4 Results

4.4.1 Macrophage CSE expression in response to acute pancreatitis and molecular mechanisms

Previous reports have shown increased CSE expression in RAW 264.7 murine macrophage cells in response to LPS stimulation using qPCR and western blotting (55, 201). I therefore chose to use this cell line to investigate the response of macrophages to pancreatitis with regards to CSE expression. Initial experiments were conducted using LPS as the stimulant in order to optimise experimental methods and inhibitor concentrations. In the following experiments, qPCR was used to measure CSE expression in RAW 264.7 cells due to its sensitivity, specificity and quantitative ability.
4.4.1.1 Measuring CSE expression in RAW 264.7 macrophage cells

A previous report has shown a dose dependent increase in RAW 264.7 CSE expression in response to LPS with the highest dose of 1 µg/mL (55), therefore this concentration was used for these experiments. RAW 264.7 cells stimulated with 1 µg/mL LPS showed increased CSE expression over corresponding saline control at 6, 12 and 24 h post stimulation using qPCR with β-actin as the reference gene (Figure 4.1). Significant differences between saline and LPS treatment were found at 6 and 24 h (p<0.05). The largest increase was observed at 24 h post stimulation, ~ 9 ± 2 fold increase, and was used for further experiments to elucidate the pathway by which LPS upregulates CSE expression.

![Graph showing CSE expression in LPS stimulated RAW 264.7 cells](image)

**Figure 4.1 Time course CSE expression in LPS stimulated RAW 264.7 cells.** qPCR shows increased expression of CSE in RAW 264.7 cells over time when stimulated with 1 µg/mL LPS over time. Bars represent mean ± S.E.M. (n=3) * denotes significant differences between LPS and saline treatment (p<0.05)

4.4.1.2 Molecular mechanisms of LPS stimulated CSE expression in RAW 264.7 cells

Having corroborated previous findings and established a method of measuring CSE expression in RAW 264.7 cells, the next step was to investigate the pathway leading up to increased CSE expression in macrophages. The pathway of interest was PI3K-Akt-sp1 and LPS was used as the stimulant in order to test the effect of the pathway inhibitors. RAW264.7 cells were pretreated with pharmacological inhibitors of sp1 (Mithramycin A), PI3K (LY294002) and Akt (Triciribine) at concentrations previously reported in this cell line (371-374). There was a dose dependent reduction of LPS stimulated increase
of CSE expression using all three inhibitors (Figure 4.1a–c). This shows that the PI3K-Akt-sp1 pathway is involved in the upregulation of CSE expression in LPS stimulated macrophages and the inhibitor concentrations were effective. The highest inhibitor concentration dose was used for further experiments.

![Figure 4.1: Pathway of LPS mediated CSE upregulation in RAW 264.7 cells. qPCR of CSE expression in LPS stimulated RAW 264.7 cells using β-actin as a reference gene. Relative expression of LPS and inhibitor treated groups were compared to untreated vehicle. Cells were treated with various inhibitors 1 h prior to and throughout LPS (1 µg/mL) stimulation. Mithramycin A, LY294002 and Triciribine showed dose dependent inhibition of LPS mediated CSE expression in RAW 264.7 cells (A – C). Bars represent mean ± S.E.M. (n=3). # denotes significant difference over vehicle untreated cells while * denotes significant difference over vehicle LPS treated cells (p<0.05).](image)

4.4.1.3 CSE expression in RAW 264.7 macrophage cells in response to stimulation with plasma of control mice or mice with acute pancreatitis

In order to observe the effect of pancreatitis on macrophage activation and CSE expression in vitro, RAW 264.7 cells were stimulated with plasma harvested from mice induced with acute pancreatitis. Ideally, peritoneal ascitic fluid would be the choice of stimulant given the location of the pancreas and the presence of resident peritoneal macrophages making it more pathophysiologically relevant. However plasma was chosen as the source of stimulant over pancreatic ascitic fluid because of its larger available volume making extraction more practical and also the fact that the peritoneal fluid would eventually drain into the blood circulation via the lymphatics. The effect of plasma stimulation on RAW 264.7 was studied by measuring the cytokine TNF-α as well as chemokines MCP-1 and MIP-
2α release. These are part of the key inflammatory mediators believed to be primarily responsible for the systemic manifestations of acute pancreatitis and its associated distant organ dysfunction (301, 307). RAW 264.7 cells stimulated with plasma from pancreatic mice showed a substantial and significant increase in pro-inflammatory cytokine secretion after 24 h when compared with plasma from control mice (p<0.05) (Figure 4.3 d to f). These levels were also marked higher compared to corresponding media only controls (p<0.05) (Figure 4.3 a to c). However it is interesting to note that the MCP-1 levels in the media only controls (Figure 4.3 b) were significantly higher in the caerulein group. This suggests that substantial levels of MCP-1 is present in the plasma of pancreatic mice compared to the saline control; however these levels of MCP-1 are substantially increased upon treatment with RAW 264.7 cell, ~175 pg/mL compared to ~25 pg/mL (Figure 4.3 e). Taken together, this would suggest that plasma from pancreatic mice was able to stimulate the RAW 264.7 macrophage cell line presumably through pro-inflammatory mediators present in the plasma as a result of pancreatitis.
Figure 4.3 Cytokines released by RAW 264.7 cells following stimulation with plasma of control mice or mice with acute pancreatitis. Plasma was obtained from mice treated with saline (control) or caerulein (pancreatitis) and used to stimulate RAW 264.7 cells by culturing cells in DMEM supplemented with 10% vlv of pooled plasma for 24 h. The media was then assayed for pro-inflammatory cytokines by ELISA. Panel (A) to (C) represent media only controls that consist of plasma supplemented media without cells cultured for 24 h, panel (D) to (F) represent plasma supplemented media cultured with RAW 264.7 cells for 24 h. Plasma from caerulein mice substantially increased pro-inflammatory cytokine secretion by RAW 264.7 cells over corresponding saline controls. These levels were also markedly higher than media only controls. Bars represent mean ± S.E.M. (n=3). * denotes significant difference compared to saline group (p<0.05).

Next, I sought to determine if the RAW 264.7 cells had altered CSE expression levels in response to plasma stimulation. It was found that stimulation of cells with plasma from pancreatic mice resulted in increased CSE expression after 24 h. There was a 1.9 ± 0.12 fold increase in gene expression as determined by qPCR and this was corroborated with protein levels as determined by western blot (2.3 ± 0.5 fold increase) (p<0.05) (Figure 4.4).
Figure 4.4 CSE expression in plasma stimulated RAW 264.7 cells. Plasma was obtained from mice treated with saline (control) or caerulein (pancreatitis) and used to stimulate RAW 264.7 cells by culturing cells in DMEM supplemented with 10% v/v of pooled plasma for 24 h. Cells were harvested and measured for CSE expression by western blot (A) and (B) and qPCR (C). Bars represent mean ± S.E.M. (n=3). * denotes significant difference compared to saline group (p<0.05).

Finally, RAW264.7 cells were pre-treated with the same pharmacological inhibitors used in the LPS stimulation experiments to determine if a similar signalling pathway is involved in the upregulation of CSE expression in response to pancretitic plasma stimulation. The concentration of inhibitors used were 2 μM Mithramycin A, 20 μM LY294002 and 0.4 μM Triciribine based on the dose dependent LPS stimulation experiments (Figure 4.2). Pancreatic plasma mediated upregulation of CSE expression in RAW264.7 cells was attenuated with the sp1 and PI3K inhibitors, Mithramycin A and LY294002. However there was a substantial potentiation of CSE upregulation following pre-treatment with the Akt inhibitor, Triciribine.
These results suggest CSE expression is significantly upregulated in macrophages during pancreatitis. The pathway leading to this increased expression is positively regulated by PI3K and Sp1 and is negatively regulated by Akt.

4.4.2 Macrophage CBS expression in response to LPS and acute pancreatitis

CBS is another hydrogen sulfide synthesizing enzyme that is present in the peripheral tissue. Therefore it would be of interest to determine CBS expression in RAW 264.7 cells in response to an inflammatory stimulus. Basal CBS gene expression along with LPS and plasma stimulated cell expression were determined using PCR by observing products on an agarose gel (Figure 4.6). There was no detectable
expression of CBS in the untreated or plasma and LPS stimulated cells. The positive control using mouse liver template showed the expected splice variants of CBS at 172 and 214 bp. Due to the lack of CBS expression, the focus remained on CSE.

![Image of PCR gel showing CBS and β-actin expression](image)

**Figure 4.6 CBS expression in RAW 264.7 cells.** A PCR was performed to detect basal as well as plasma and LPS stimulated CBS expression in RAW 264.7 cells. PCR was carried out for 30 cycles using mouse liver template as a positive control (+ve) and β-actin as a reference gene. CBS expression was not detectable in basal (Untreated) or plasma (Saline and Caerulein) and LPS stimulated cells.

### 4.5 Discussion

Understanding the molecular and cellular basis of diseases is vital for dissecting the mechanisms of disease pathogenesis and for designing appropriate and effective treatments. The molecular mechanisms involved in regulating macrophage CSE expression are poorly studied; there are only two very recent publications to date that describe the pathway involved in LPS (375) and statin (376) mediated upregulation of CSE. In this chapter, I have investigated the molecular mechanism leading to macrophage CSE expression in response to the classical endotoxin activator, LPS and extended these findings to stimulation with plasma from pancreatitic mice. I chose to study the PI3K signalling pathway as this has been reported to both positively and negatively regulate the expression of pro-inflammatory mediators in LPS stimulated macrophages (373, 377), and could therefore account for the dual roles of CSE/endogenous hydrogen sulfide reported in macrophages (201, 249).

The findings of this chapter highlight the increased expression of CSE in macrophages under inflammatory conditions such as LPS stimulation as well as pancreatitis. These results represent the only report that links macrophage CSE upregulation to an *in vivo* model of inflammatory disease. This
shows that CSE/endogenous hydrogen sulfide may play a role in macrophage function and therefore the inflammatory response at large. This chapter has also elucidated the molecular mechanisms leading up to CSE overexpression in macrophages in response to LPS and pancreatitis involving PI3K, Akt and sp1 which have not been previously shown. The LPS mediated PI3K-Akt-sp1 mechanism described here ties in with recent findings of LPS mediated TLR4-p38 and TLR4-NFκB dependent upregulation of CSE in macrophages (379,380) to suggest a TLR4-PI3K-Akt-p38-sp1 pathway.

4.5.1 Molecular mechanisms leading to LPS induced CSE upregulation in macrophages

Genetic characterization of the murine CSE cDNA in transfected HEK-293 and Cos-7 cell lines revealed several transcription factor binding sites in the core promoter region, among them sp1 and n-myc were found to play major roles in the basal transcription (16). LPS is known to activate sp1 in macrophages and has been shown to regulate the expression of pro-inflammatory related proteins such as histidine decarboxylase (HDC) (371), MIP-2 (378) and TNF-α (379) as well as anti-inflammatory ones such as IL-10 (380, 381). Therefore I had chosen investigate the involvement of sp1 in the regulation of macrophage CSE expression. Here, I have also shown that sp1 is a key transcription factor responsible for LPS mediated CSE upregulation in the murine macrophage cell line, RAW264.7 (Figure 4.2).

Classical macrophage activation by LPS occurs via the TLR-4 receptor and activation of two major intracellular signalling pathways involving the mitogen-activated protein kinases (MAPKs) and NFκB respectively leading to the expression of pro-inflammatory cytokines and effector molecules (382). TLR-4 activation by LPS has also been shown to activate phosphoinositide 3-kinase (PI3K) signalling in macrophages although it is still unclear if this positively or negatively regulates the expression of pro-inflammatory mediators in LPS stimulated macrophages (373, 383). PI3K signalling has been shown to phosphorylate and activate sp1 via PI3K-Akt and PI3K-PKC-ζ (reviewed by (384)) and this has been demonstrated in IFN-γ and LDL stimulated macrophages (385, 386). Here, I have shown that CSE upregulation in LPS stimulated RAW264.7 cells is dependent on PI3K signalling and its downstream effector kinase, Akt (Figure 4.2). Taken together, these results suggest that the LPS stimulated expression of CSE in RAW264.7 cells occurs via a PI3K-Akt-sp1 dependent pathway (Figure 4.7).
These results are supported by recent findings showing a TLR4-p38 and TLR4-NFκB mediated upregulation of CSE expression in LPS stimulated macrophages (322). TLR4 is known to stimulate PI3K signalling in LPS stimulated macrophages (379,380) and this has been shown to activate both p38 (381,382) as well as NFκB (380) via Akt; phosphorylation of p38 has also been shown to activate sp1 leading to gene expression in macrophages (383). Taken together this suggests that LPS stimulation of macrophage leads to CSE upregulation via a TLR4-PI3K-Akt-p38-sp1 signalling pathway.

4.5.2 CSE upregulation in panreatitic plasma stimulated macrophages and the molecular mechanisms involved

The role of CSE and endogenous hydrogen sulfide in macrophages are still poorly understood within the context of pancreatitis. An in vitro method of studying the macrophage activation in pancreatitis involves the application of peritoneal ascitic fluid from experimental severe acute pancreatitis in rats onto cultured macrophages (310). This has been shown to stimulate macrophages by activating NFκB and increasing TNF-α production (310, 311). The caveat of using PAAF is the limited quantities available particularly in smaller experimental animals such as mice; therefore I elected to use pooled plasma instead, as the peritoneal fluid would eventually drain into the circulation via the lymphatics. The drawback of using plasma however is that it contains ~ 10 times less pancreatic enzymes and cytokines than PAAF following pancreatitis (297).

Therefore initial experiments were carried out to ascertain the ability of pancreatitis derived plasma to stimulate macrophages in vitro. RAW264.7 cells cultured in serum free media supplemented with 10 % v/v plasma from pancreatitic mice showed a dramatic increase of up to several orders in MCP-1, MIP-2α and TNF-α release over a 24 h period in comparison to plasma from control mice (Figure 4.3). These molecules are released by activated macrophages (387, 388) and represent key inflammatory mediators that are believed to be primarily responsible for the systemic manifestations of acute pancreatitis and its associated distant organ dysfunction (301, 307). Having established the ability of pancreatitic plasma in stimulating RAW264.7 cells in vitro, I then employed this model as an approximation to ascertain the CSE expression status of macrophages during pancreatitis. The results show about ~2 fold increase in RAW264.7 CSE expression in response to plasma stimulation from pancreatitic mice compared to control mice over a 24 h period (Figure 4.4). This increase could
potentially be higher bearing in mind that plasma levels of pro-inflammatory mediators are substantially lower than in the PAAF in pancreatitis (297).

Using the optimal inhibitor concentrations determined from the LPS stimulation experiments (Figure 4.2), I have shown that increased CSE expression in RAW264.7 cells in response to plasma stimulation from pancreatitic mice was dependent on sp1 and PI3K activation (Figure 4.5). These findings were similar to the LPS mediated increase however in contrast, the Akt inhibitor Tricirine potentiated plasma stimulated increase in CSE expression. This unexpected outcome raises two issues; the ability of Akt to mediate both up and downregulation of CSE expression and the differential regulation of CSE expression by PI3K and Akt respectively. Apart from phosphorylating and activating sp1 (reviewed by (384)), Akt is also known to phosphorylate and inactivate glycogen synthase kinase 3 (GSK3) (389), which is constitutively active in unstimulated cells and phosphorylates N-myc among other proteins, to promote GSK3 degradation and increase the availability of N-myc (390). N-myc has been shown to be a repressor of CSE expression (16) and is found to be constitutively expressed in RAW264.7 cells (391). Taken together, the Akt-GSK3-N-myc pathway could be a method by which CSE expression is repressed or downregulated by activation of Akt, and this explains how Akt activation could both up and downregulate CSE expression. PI3K activation leads to the activation of a large number of proteins and lipids that contain a plekstrin homology domain (reviewed in (392)). Among them is phosphoinositide-dependent kinase 1 (PDK1) which is capable of phosphorylating numerous proteins including Akt and PKC-ζ. As mentioned above, both Akt and PKC-ζ are capable of phosphorylating and activating sp1 (reviewed in (384)). Therefore there are and could be other mechanisms by which PI3K could activate sp1 independent of Akt owing to the diverse signalling targets of PI3K and Akt (reviewed in (392)). In the case of pancreatitic plasma stimulated RAW264.7 cells, there appears to be a simultaneous upregulation of CSE expression via PI3K and downregulation by Akt signalling. In comparison to LPS stimulation, the plasma from pancreatitic mice consists of a complex combination of molecules that could contain both pro and anti-inflammatory cues. This could explain the observed divergent roles of PI3K and Akt on CSE expression possible mediated by separate signalling events from the plasma and the net effect of this differential regulation is the increased expression of CSE in RAW264.7 cells. This is summarised in Figure 4.7.
Figure 4.7 Molecular mechanisms of LPS and pancreatitic plasma stimulated upregulation of CSE expression in RAW 264.7 cells. This diagram summarises this chapter’s findings on the pathway leading up to LPS and pancreatitic plasma stimulated CSE expression in macrophages. LPS was shown to elicit CSE expression via PI3K-Akt-sp1. Plasma from pancreatitic mice however appears to elicit a complex activation of macrophage signalling resulting in a net increase of CSE expression via a PI3K-sp1 pathway. This does not involve the classical PI3K signalling partner, Akt, which was shown to negatively regulate CSE expression instead. This could possibly occur via phosphorylation of GSK-3 resulting in activation of N-myc which is a known transcriptional deactivator of the CSE gene.

4.5.3 CBS expression in macrophages

CBS is another major endogenous hydrogen sulfide synthesizing enzyme apart from CSE. There have been conflicting reports on the expression of CBS in macrophages; two studies have reported undetectable CBS levels in murine primary peritoneal macrophages as well as the RAW264.7 macrophage cell line (55, 321), while one report has shown basal CBS expression in the human THP-1 macrophage cell line (205). In order to determine the relevance of CSE as an inducible source of
endogenous hydrogen sulfide in macrophages, I sought to detect CBS expression in RAW264.7 cells under basal and stimulated conditions. Using PCR, there was no detectable expression of CBS in unstimulated cells or upon 24 h stimulation with LPS and plasma from pancreatitic mice (Figure 4.6). These results are in agreement with previous studies (55, 321) and shows that CSE is the main hydrogen sulfide synthesizing enzyme present and upregulated in macrophages upon stimulation and could therefore play an important role in macrophage inflammatory response.
5 The effect of CSE gene deletion in caerulein-induced pancreatitis in the mouse

5.1 Introduction

5.1.1 CSE dysregulation and endogenous hydrogen sulfide synthesis in pathological conditions

CSE is classically known for its role in the transsulfuration pathway for the $\alpha,\gamma$-elimination of cystathionine to yield cysteine, $\alpha$-ketobutyrate and ammonia (393). However, CSE is also capable of generating hydrogen sulfide as a byproduct through cysteine-dependent $\beta$-reactions and homocysteine-dependent $\gamma$-reactions (21). In experimental models the induction of pathological conditions such as sepsis (189), LPS-induced endotoxaemia (44, 375, 394), hepatic ischemia-reperfusion injury (395), burn injury (45), renal injury (217), colitis (328), gall bladder inflammation (329), pancreatitis (69, 322) and chronic rhinitis (396) are associated with upregulation of CSE expression in the target organ of the respective disease. Increased CSE expression has also been detected in immune response cells such as macrophages when stimulated with LPS (55, 201, 375). Not all inflammatory conditions are associated with upregulation of CSE and endogenous hydrogen sulfide; asthma (397, 398), gastropathy (327), hepatotoxicity (223), kidney ischemia reperfusion injury (399) and ox-LDL stimulated macrophages (321) have shown a down-regulation of CSE instead along with the reduction in endogenous hydrogen sulfide synthesis (223, 397).

5.1.2 The role of CSE and endogenous hydrogen sulfide in the inflammatory response

Recent insights into the physiological aspects of hydrogen sulfide and its direct mediated effects have revealed potential ways in which it could influence an inflammatory response. Hydrogen sulfide has been shown to be a physiologically relevant vasodilator (18, 80), cytoprotectant (reviewed by (191)) via its antioxidant and anti-apoptotic roles as well as a pro-nociceptor which could lead to neurogenic inflammation (105, 112, 113) (discussed in further detail in section 1.3). Hydrogen sulfide has also been reported to directly modify thiol containing proteins through $\text{s}$-sulfhydration, function as a direct antioxidant and bind to metal ion containing proteins. These direct interactions have been shown to modulate activity of key inflammatory mediators such as reactive oxygen/nitrogen species,
transcription factors NFκB and Nrf2, neutrophilic myeloperoxidase enzyme as well as the renin-angiotensin system (discussed in further detail in section 1.4).

The effect of CSE/endogenous hydrogen sulfide dysregulation on the inflammatory response has been the subject numerous studies. The use of a selective pharmacological CSE inhibitor, PAG, in animal models of disease have reported both pro (44, 45, 66, 189, 216-219, 400) and anti (124, 187, 220-223, 401) inflammatory effects. Increased CSE expression/endogenous hydrogen sulfide has been associated with both increased and decreased circulating inflammatory cytokines/chemokines (44, 66, 200, 217, 223), pro and anti-apoptotic effect (69, 124), increased and decreased oxidative stress (218, 223) as well as increased and decreased iNOS expression and NO production (55, 201, 222). These conflicting results could be due to several factors such as the difference between the pathology of diseases studied, the dosage regime of PAG as well as route of administration and that PAG itself is not specific towards CSE and targets other pyridoxal-5-phosphate dependent enzymes (discussed further in introduction 1.5.4). Therefore an alternative and more specific approach towards inhibiting CSE/endogenous hydrogen sulfide would be beneficial in enhancing our understanding of the role of CSE/endogenous hydrogen sulfide in inflammation.

5.1.3 Caerulein induced acute pancreatitis: an inflammatory response model

Pancreatitis is a clinical condition that is thought to stem from the intracellular activation of digestive enzymes and autodigestion of the pancreas (298) and is regarded as a sterile inflammatory response in the initial stages; intracellular contents released from damaged necrotic acinar cells into the extracellular space serve as DAMPs that trigger inflammation and is a key determinant of further pancreatic injury, remote organ injury, and disease resolution in experimental models (276). This non-pathogenic destruction of pancreatic parenchyma rapidly elicits an inflammatory reaction at the site of injury and has the potential to amplify this localised process resulting in a generalised systemic inflammatory response. Sterile inflammation is very similar to microbial induced inflammation, both instances result in the recruitment and activation of the innate immune response cells as well as production of pro-inflammatory cytokines and chemokines via PRR mediated pathways (402).
The benefit of using a sterile inflammatory model of disease provides the opportunity to study the inflammatory response independent of interaction with a pathogen, this is particularly pertinent in the field of hydrogen sulfide. Most, if not all bacteria have orthologs of mammalian CBS, CSE or 3MST and the capacity for hydrogen sulfide synthesis (403, 404). Certain bacteria are also capable of producing hydrogen sulfide by using sulfate as the final electron acceptor in the respiration chain thus reducing it to hydrogen sulfide (405). In an inflammatory response involving bacteria as the pathogen such as sepsis, there is a possibility of interplay between the effect of bacterial derived hydrogen sulfide on the host response and vice-versa. For example hydrogen sulfide has been shown to be a universal bacterial defence against antibiotics induced oxidative stress (403) and on the other hand, hydrogen sulfide metal chelating ability mimics the innate defence system of employing the chelator protein, calprotectin as means to starve the invading bacteria of essential ions required for growth (406, 407). However as described in section 1.6.1.3, acute pancreatitis is also associated with bacterial infection stemming from intestinal permeability. This however, is mostly associated with the severe form of acute pancreatitis. In animal models of acute pancreatitis, severe acute models such as sodium taurocholate and sodium deoxycholate infusion as well as bilio-pancreatic duct ligation have shown to induce bacterial and endotoxin translocation as early as 4 to 18 hours post surgery (408-411). These models are also associated with mortality. In contrast the caerulein model involving hyperstimulation of the pancreatic acinar cells induces a milder form of acute pancreatitis; bacterial as well as endotoxin translocation was reported 24 hours post induction and was found to be less pervasive in comparison to the severe acute models (411, 412). It is also not associated with mortality. Therefore, the use of caerulein-induced pancreatitis as a model of inflammation would be useful to investigate the role of CSE/endogenous hydrogen sulfide in the inflammatory response as it is minimally invasive, involves non-pathogenic triggers of inflammation, is severe enough to initiate a systemic inflammatory response and is associated with minimal or no secondary bacterial infection. Currently, there have been several studies using PAG to determine the role of CSE/endogenous hydrogen sulfide in pancreatitis. Although these studies differed in terms of method of induction, the dosage of PAG was very similar. Three of these studies employed a minimally invasive caerulein hyperstimulation method of induction that involves hourly intraperitoneal injections of caerulein for ten hours in mice resulting in acute pancreatitis (66, 244, 318). The remaining two studies adopted a
surgical method of retrograde sodium taurocholate infusion into the pancreatobiliary duct in rats causing severe acute pancreatitis (69, 322). In all the studies, animals were administered 50-100 mg PAG per kg body weight intraperitoneally either before (30 min-1 h) or 1 h after the initial induction of pancreatitis. All five studies reported attenuation in disease severity following PAG treatment, this was associated with reduction in pancreatic and circulating inflammatory cytokines/chemokines (318) as well as increased acinar cell apoptosis as opposed to necrosis as a more favourable and contained form of cell death (69). Although PAG has proved to be a valuable resource to investigate the role of hydrogen sulfide synthesised by CSE in inflammation, possible actions of PAG unrelated to CSE inhibition lead to criticism of all studies where PAG has been used.

5.2 Aims

The previous chapter has shown increased pancreatic CSE expression and hydrogen sulfide synthesising activity in response to pancreatitis. Macrophages stimulated with plasma of pancreatic mice also showed increased CSE expression over the corresponding control. This chapter will focus on determining the effect of CSE/endogenous hydrogen sulfide in inflammation using caerulein induced acute pancreatitis in mice as the model of disease. To do so, I compared the response of wild type and CSE deficient mice to pancreatitis in terms of disease severity (characterised by pancreatic and lung neutrophil infiltration as evidenced by MPO activity and pancreatic damage as evidenced by acinar cell necrosis and oedema), pancreatic sulfide production and pancreatic inflammatory mediator levels (cytokines, chemokines, eicosanoids and NFκB). The use of knockout mice would eliminate the use of pharmacological inhibitors, along with their potential drawbacks, to gain a more definitive insight to the role of endogenously synthesized hydrogen sulfide in inflammation. There will also be a secondary focus comparing the effect of in vitro stimulated pancreatic acinar cells isolated from wild type and CSE deficient mice to investigate the role or CSE/endogenous hydrogen sulfide in the progression of this disease at the acinar cell level by comparing their ability to release chemotactic molecules (MCP-1 and LTB₄) in response to caerulein stimulation.
5.3 Materials and methods

5.3.1 Caerulein induction of acute pancreatitis in mice

Plasma was obtained from mice induced with acute pancreatitis mice using caerulein as described below. Plasma was pooled from individual mice treated with saline or caerulein and frozen in aliquots to make up a batch of working plasma to treat RAW 264.7 cells.

All animal experimentation were approved by the Animal Ethics Committee of the University of Otago (Protocol C57/11) and performed according to the guidelines. Caerulein was obtained from Bachem (Bubendorf, Switzerland) and reconstituted in sterile saline to a final concentration of 1 mg/100 mL and frozen in 1.5 mL aliquots. WT and CSE KO C57BL6 mice (male, 20–25 g) were assigned randomly to control or experimental groups. WT mice were obtained from the Christchurch Animal Research Area, and the CSE KO mice were a gift from Dr. Ishii Isao from the Graduate School of Pharmaceutical Sciences, Keio University, Japan. A total of 32 animals was used, WT saline (n = 6), KO saline (n = 4), WT caerulein (n = 11), and KO caerulein (n = 11). Animals were given hourly intraperitoneal (i.p.) injections of normal saline (0.9% wt/vol NaCl) or caerulein (50 μg/kg) for 10 h, as described previously (305, 361). For pain relief, all mice were given 3 subcutaneous (s.c.) doses of buprenorphine (0.2 mg/kg) per hour before treatment, as well as 3 h and 7 h into the treatment. One hour after the last caerulein/saline injection, animals were sacrificed by an i.p. injection of sodium pentobarbital. Blood samples were drawn from the right ventricle using heparinized syringes and centrifuged (1,000 g for 10 min, 0–4 °C). Thereafter, plasma was aspirated and stored at -80°C for amylase measurement. Random cross sections of the pancreas and lungs were fixed in 4% wt/vol neutral phosphate-buffered formalin for subsequent histological analysis. Samples of pancreas and lung were also weighed out immediately on preweighed boats for determination of water content. The remaining samples of pancreas and lung were stored at -80 °C for subsequent measurement of tissue myeloperoxidase (MPO) activity, estimation of CSE expression by Western blotting, as well as measurement of cytokines and prostaglandin E₂ (PGE₂) levels by ELISA.
5.3.2 Primary pancreatic acinar fragment preparation and treatment

The media use for isolating primary pancreatic acinar cells consisted of DMEM supplemented with 0.1% w/v BSA, 0.05% w/v trypsin inhibitor and oxygenated with 95% oxygen and 5% CO₂ before use. The method of isolation was based on Bruzzone et al (362). Freshly removed pancreas were placed in ice cold media to rinse off excess blood and then transferred to a 1.5 mL microfuge tube on ice containing media supplemented with collagenase type IV at a final concentration of ~400 U/mL. The pancreas was minced finely using scissors to pieces < 1 mm in diameter. The minced pancreas was then transferred to a glass vial, topped up with collagenase solution, sealed tightly and in a 37 °C shaking water bath for 10 mins. After that the vial was shaken vigorously by hand for 1 min and placed back into the bath for a further 5 mins after which it was vigorously shaken by hand for a final 30 s. The digest was then briefly triturated with a large orifice plastic pasteur pipette and layered onto a 4% BSA solution in a 15 mL tube. This was then centrifuged at 50 g for 2 mins, the collagenase and BSA layers were discarded and the cells were rinsed with fresh media 2 times by centrifuging at 50g for 70 s each time. The pellet was the triturated in fresh media and the top layer of fine tissue fragments as the bigger clumps settled was transferred to a fresh tube to be used for experiments. These fine fragments consisted of cell clusters of 5 to 20 cells and had viability of more than 90 % as determined by trypan blue exclusion.

The final pancreatic fragments were distributed into 1.5 mL microfuge tubes and treated with either saline or caerulein at a final concentration of 100 nM. They were incubated at 37 °C on a rotator for 60 mins after which they were harvested by centrifugating at 1000 g for 5mins. The supernatants were assayed for MCP-1 and LTB₄ levels by ELISA and corrected for total protein content in the pellet.

5.3.3 Tissue preparation and processing for histological analysis

Tissue samples were fixed overnight in 4% wt/vol neutral phosphate-buffered formalin and sent to the Christchurch hospital histopathology department to be processed and set into paraffin blocks. 4μm thick formalin fixed paraffin embedded tissues sections were cut using a Leica microtome and immobilized onto superfrost coated glass slides (Menzel-Glaser, Germany). Sections were left to air
dry and subsequently stained for hematoxylin/eosin by the Christchurch hospital histopathology department or used for immunohistochemistry.

5.3.4 Plasma amylase activity

Amylase activity was measured using a standard kinetic spectrophotometric assay. 5 μL of plasma samples were incubated with 200 μL of the ready to use assay mixture containing Ethyldene-pNP-Glucose-7 (EPS) and α-glucosidase (Thermo Fisher Scientific, USA). EPS is the substrate for α-amylase, upon cleavage the resulting smaller fragments are acted upon by the α-glucosidase to yield the final chromophore measured at 405nm. Measurements were taken at 60s intervals for a total of 4 min. The resulting change in absorbance from the linear portion of the curve was used to calculate amylase activity as described by the manufacturer’s protocol.

5.3.5 Tissue myeloperoxidase measurement

Leukocyte sequestration in pancreas and lung was quantified by measuring tissue myeloperoxidase (MPO) activity. Tissue samples were thawed and homogenised in 20 mM phosphate buffer (pH 7.4) (~50mg/mL) supplemented with protease inhibitors (Halt by Thermo Scientific Pierce Protein Biology, Rockford, IL, USA) on ice using a Labserv homogeniser. Homogenates were then centrifuged (10,000 g, 10 min, 4°C) and the resulting pellet resuspended in 50 mM phosphate buffer (pH 6.0) containing 0.5% w/v hexadecyltrimethylammonium bromide (Sigma). The suspension was subjected to four cycles of freezing and thawing and further disrupted by sonication on ice (40 seconds). The samples were then centrifuged (10,000 g, 5 min, 4°C) and the clear supernatant was used for the MPO assay. The reaction mixture consisted of the supernatant (50 μl) and stabilized tetramethylbenzidine and hydrogen peroxide mixture (R&D Systems, USA) (reagent volume: 50 μl). This mixture was incubated at room temperature for ~ 10 mins for the colour to develop and the reaction was terminated with 50 μl of 2N hydrogen sulfideO₄. The absorbance was measured at 450 nm with a 570 nm correction. This absorbance was then corrected for the protein content of the tissue sample using the Bradford assay and results were expressed as fold increase over control.
5.3.6 **Morphological examination of pancreatic damage**

4 μm pancreatic sections were stained with hematoxylin & eosin and mounted by the Christchurch Hospital Histopathology Department. Two sections were prepared for each pancreas and two random images were taken from each section at 20x magnification. Pancreatic pathology was assessed in a blinded manner based on the previously described Schmidt Scoring Criteria (413). Assessment was based on three criterion of edema, acinar cell necrosis and leukocyte infiltration. Edema was graded on expansion of interlobular and interacinar septae and its distribution (focal or diffused). The extent of acinar cell necrosis was determined by both number of necrotic cells per high powered field and its distribution (focal or diffused). Leukocyte infiltration was scored based on the number of cells present per high powered field. Each of these criterion was then graded from 0 to 4 with increments of 0.5 based on the severity as described in detail by Schmidt et al (413).

5.3.7 **Pancreatic hydrogen sulfide synthesizing activity**

hydrogen sulfide synthesizing activity in pancreatic homogenates was measured with a modified protocol based on methods described previously (365). ~50mg pancreatic tissue was homogenised in 50 mM ice-cold potassium phosphate buffer (pH 7.4) using a Labserv homogeniser. The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.4), L-cysteine (20 μl, 10 mM), pyridoxal 5’-phosphate (20 μl, 2 mM), saline (30 μl), and 11% w/v tissue homogenate (430 μl). The reaction was performed in tightly parafilm-sealed microfuge tubes (with lids taken off) and initiated by transferring the tubes from ice to a water bath at 37°C. After incubation for 30 min, 1% w/v zinc acetate (250 μl) was injected in to trap evolved hydrogen sulfide followed by 10% v/v trichloroacetic acid (250 μl) to denature the protein and stop the reaction. Subsequently, N,N-dimethyl-p-phenylenediamine sulfate (20 μM; 133 μl) in 7.2 M HCl was added, immediately followed by FeCl$_3$ (30 μM; 133 μl) in 1.2 M HCl. Samples were left to incubate at room temperature in the dark for 20 min and subsequently pelleted. The absorbance of the clear supernatant was measured with a 96-well microplate spectrophotometer at 670 nm. The hydrogen sulfide concentration was calculated against a calibration curve of Na$_2$S. Results were then corrected for the protein content of the tissue sample determined by the Bio-rad DC protein assay and are expressed as nmole hydrogen sulfide formed/mg protein.
5.3.8  **Cytokine and eicosanoid measurement by ELISA**

Pancreatic IL-6, MCP-1, MIP-2α and PGE$_2$ levels as well as pancreatic acinar cell supernatant MCP-1 and LTB$_4$ levels were measured using ELISA Duo Set kits from R&D Systems (USA) according to the manufacturer’s protocol. Pancreatic homogenates were prepared by homogenizing ~50 mg tissue in 1 mL of 20 mM sodium phosphate buffer (pH 7.4) supplemented with protease inhibitors (Halt by Thermo Scientific Pierce Protein Biology, Rockford, IL, USA) on ice. Homogenates were spun at 30,000 g for 15 mins at 4°C and 100 µL of the clear supernatant was used for each assay. Each kit consisted of a capture and biotin conjugated detection antibody pair, standards and streptavidin-HRP conjugate. ELISA specific plates (Corning, USA) were first coated with capture antibody in PBS for 1 h at RT overnight. Plates were then decanted, washed with PBST (0.05% w/v) and blocked with 300 µL of BSA (1% w/v) for 1 h at RT followed by washing and addition of 100 µL samples or standards. After 2 h incubation, plates were decanted, washed followed by addition of 100 µL biotin conjugated detection antibody. After 2 h incubation, plates were decanted, washed followed by addition of 100 µL streptavidin conjugated HRP. After a 30 min incubation, plates were decanted, washed followed by addition of 100 µL substrate reagent (R&D Systems, USA). Plates were incubated at RT for up to 20 mins to allow the colour to develop and the reaction was terminated by addition of 50 µL sulfuric acid. Plates were read at 450 nm on a spectrophotometer (Spectramax, Molecular Devices, USA) with a 570 nm correction. Cytokine and eicosanoid levels were corrected for total protein and expressed as pg or ng per mg of protein.

5.3.9  **Immunohistochemical detection of NFκB translocation in pancreatic acinar cells**

Staining was performed on 4µm thick formalin fixed paraffin embedded tissues sections using the rabbit specific HRP/DAB (ABC) Detection IHC Kit (Abcam, Cambridge UK). Sections were deparaffinized in xylene and rehydrated through a graded alcohol series (described in section 3.3.6) followed by an antigen retrieval step by incubating in a 10 mM Sodium citrate, 0.05% Tween 20, pH 6.0 solution at 97°C for 20 mins. Sections were allowed to cool at room temperature for 20 mins followed by immunostaining as described in the manufacturers’ protocol. Briefly, sections were serum/protein blocked for 30 mins at RT followed by an overnight incubation at 4°C with primary antibody; rabbit
anti- NFκB p65 (Abcam, Cambridge UK) diluted 1:500 in TBST (0.025% w/v)) with 1% BSA. This was followed by a hydrogen peroxide block for 10 mins, secondary antibody incubation for 30 mins, streptavidin incubation for 20 mins, DAB substrate incubation for 1 min, rinse in water, brief dip in diluted hematoxylin, and final rinse in water. TBST (0.025% w/v) was used as the wash buffer between reagents. Sections were then dehydrated through an alcohol series followed by xylene and mounted. NFκB translocation was determined by positive staining of the acinar cell nuclei for the NFκB p65 subunit. Four random fields of view (FOV) were taken from each section with a 20x objective lens and the number of positively stained nuclei was averaged from the four FOVs.

5.3.10 Statistical analysis

Data are presented as means ± S.E.M. Statistical analysis was performed using the Graphpad Prism Software version 5.03. Multiple comparisons were made using ANOVA with a Bonferonni post-hoc analysis. Comparisons of two data sets were made with an unpaired student’s t-test. A p value of less than 0.05 was considered significant.

5.4 Results

5.4.1 Pancreatic injury in acute pancreatitis

Both WT and CSE KO mice showed typical effects of pancreatic injury following caerulein induced pancreatitis. They were characterized by pancreatic oedema, leukocyte infiltration, acinar cell necrosis and increased plasma amylase activity (413).

The WT mice treated with caerulein showed significantly higher plasma amylase activity compared to the saline treated WT mice with means of 11351 ± 688 U/L and 1886 ± 117 U/L, respectively (p<0.05) (Figure 5.1 a). Pancreatic MPO activity was used as a measure of neutrophil infiltration and this was also higher in the WT caerulein mice with a mean fold increase of 19.7 ± 2.1 over the corresponding saline control (p<0.05) (Figure 5.1 b). Using the wet-to-dry weight ratio of the pancreas as a measure of oedema, the WT caerulein group showed a significantly higher level of oedema compared to the WT saline group with mean ratios of 5.0 ± 0.5 and 3.5 ± 0.1, respectively (p<0.05) (Figure 5.1 c).
The CSE KO mice treated with caerulein also showed higher levels of pancreatic injury in comparison to its saline treated control. There was a significantly higher level of plasma amylase in the caerulein treated CSE KO mice with a mean of 6731 ± 514 U/L compared to the CSE KO saline control with a mean of 2163 ± 229 U/L (p<0.05) (Figure 5.1 a). Pancreatic MPO levels of caerulein treated CSE KO mice were also significantly elevated with an 11.8 ± 1.6 fold increase over the corresponding saline treatment (p<0.05) (Figure 5.1 b). Although the pancreatic water content was elevated, this increase was not found to be significant (Figure 5.1 c).

These increases in pancreatic injury were however, found to be less severe in the CSE KO mice compared to the WT. The KO caerulein group had significantly lower pancreatic MPO and plasma amylase activity than the WT caerulein group (p<0.05) (Figure 5.1 a-b). Pancreatic oedema, as measured by wet-to-dry ratios, was slightly elevated but not significantly different between the caerulein treated KO mice and saline treated KO mice (Figure 5.1 c).

![Figure 5.1 Pancreatic injury in acute pancreatitis. Shows the effect of caerulein induced acute pancreatitis on A) plasma amylase levels, B) changes in pancreatic MPO activity and C) pancreatic oedema. Bars represent mean ± S.E.M. (n=4-11). * denotes significant difference between saline and caerulein treated mice of each mouse strain (p<0.05). # denotes significant difference between each mouse strain with the same treatment (p<0.05).](image)

Evidence of pancreatic oedema, leukocyte infiltration as well as acinar cell necrosis was present in pancreatic sections stained with hematoxylin and eosin (Figure 5.2). Semi quantitation of these parameters according to scoring method developed by Schmidt et al (413) corroborated with the findings described above with higher pathological scoring in the WT caerulein treated mice compared to the CSE KO caerulein treated mice (Figure 5.3). Although the WT mice had higher scoring in all three
parameters of oedema, leukocyte infiltration and acinar cell necrosis as compared to the CSE KO mice, significant difference was only detected in oedema and acinar cell necrosis (Figure 5.3 a-b).

**Figure 5.2 Haematoxylin & Eosin staining of pancreatic sections.** Representative pancreatic sections show pancreatic injury as a result of caerulein-induced pancreatitis with oedema, leukocyte infiltration and acinar cell necrosis. Haematoxylin (purple) stains nucleic acids (cell nucleus) while eosin (pink) stains protein non-specifically (cell cytoplasm and extracellular matrix).

**Figure 5.3 Semi quantitation of pancreatic damage based on H&E stained pancreatic sections.** Pancreatic injury following caerulein-induced pancreatitis was scored based on a previously established method (413) that includes number of necrotic acinar cells per field of view (A), extent of pancreatic
oedema (B) and number of leukocytes present in the tissue (C). Bars represent mean ± S.E.M. (n=4). # denotes significant difference between WT and KO mice (p<0.05).

5.4.2 Pancreatic hydrogen sulfide synthesizing activity in WT and CSE KO mice.

CSE KO mice lacked expression of the CSE protein and showed substantially significant lower pancreatic hydrogen sulfide synthesizing activity in comparison to the WT mice (saline treated group) with means of 2.87 ± 0.37 and 8.70 ± 0.69 nmol/mg/30min, respectively (p<0.05) (Figure 5.4). This suggests that CSE is the major hydrogen sulfide synthesizing enzyme present in the pancreas and is supported by previous studies using PAG (66). Following caerulein stimulation, there was a significant ~1.33 ± 8 % fold increase in hydrogen sulfide synthesizing activity in the WT mice but this was not observed in the CSE KO mice. This implies an increase in CSE mediated hydrogen sulfide synthesizing ability following caerulein stimulation which is supported by a detectable increase in pancreatic CSE expression as shown in section 3.4.1.

**Figure 5.4** Pancreatic hydrogen sulfide synthesizing activity in WT and CSE KO mice. Pancreatic hydrogen sulfide synthesizing activity of WT and CSE KO mice, KO mice had significantly lower hydrogen sulfide synthesizing activity compared to the WT. WT hydrogen sulfide synthesizing activity increased following caerulein stimulation. Bars represent mean ± S.E.M. (n=5). * denotes significant difference between saline and caerulein treated mice of each mouse strain (p<0.05). # denotes significant difference between each mouse strain with the same treatment (p<0.05).

5.4.3 Acute pancreatitis-associated lung injury

Severe acute pancreatitis often results in the development of lung injury that closely resembles the acute respiratory distress syndrome (ARDS) associated with other processes such as shock, bacteraemia, ischemia/ reperfusion, and burns (414). In this study, WT mice treated with caerulein
showed significantly more MPO activity in the lungs compared to the saline treatment with a mean fold increase of $2.36 \pm 0.94$ (p<0.05) (Figure 5.5 a), demonstrating that leukocyte infiltration occurs following caerulein induced pancreatitis. The caerulein treated KO mice showed significantly less MPO activity in the lungs compared to the WT with a mean fold increase from each saline control group of $1.30 \pm 0.10$ and $2.36 \pm 0.28$, respectively (p<0.05) (Figure 5.5 a). There was a significant difference in wet-to-dry ratios between the WT caerulein treated group compared with WT saline group with mean ratios of $4.80 \pm 0.08$ and $3.96 \pm 0.18$, respectively (p<0.05) (Figure 5.5 b). However the corresponding KO mice showed no significant differences.

**Figure 5.5 Acute pancreatitis associated lung injury.** Significantly higher lung MPO activity and water content was detected in caerulein-induced WT mice compared to saline treatment. This was not observed in the CSE KO mice. Lung MPO activity in caerulein treated KO mice was significantly less compared to the corresponding WT mice. Bars represent mean ± S.E.M. (n=4-11). * denotes significant difference between saline and caerulein treated mice of each mouse strain (p<0.05). # denotes significant difference between each mouse strain with the same treatment (p<0.05).

### 5.4.4 Pancreatic pro-inflammatory mediators

As expected WT mice treated with caerulein showed significantly higher pancreatic levels of chemokines (MCP-1 & MIP-2α), cytokine (IL-6) and eicosanoid (PGE$_2$) compared to the WT saline group (p<0.05) (Figure 5.6). This was also observed in the KO mice with the exception of IL-6 which was not significantly elevated following caerulein treatment (Figure 5.6). However the extent of these increases were significantly lower in the KO mice in comparison to WT; PGE$_2$ levels were $1.72 \pm 0.47$ ng/mg and
1.05 ± 0.05 ng/mg, MCP-1 were 83.0 ± 9.3 pg/mg and 53.0 ± 5.6 pg/mg and IL-6 were 54.1 ± 9.9 pg/mg and 21.8 ± 6.1 pg/mg in the caerulein treated WT and KO mice respectively (p<0.05) (Figure 5.6). MIP-2α did not show a significant difference between the caerulein treated WT and KO mice, however the magnitude of increase in comparison to the saline controls was lower in the KO group (Figure 5.6c).

**Figure 5.6 Pancreatic pro-inflammatory mediators.** Caerulein induced pancreatitis resulted in a significant increase in pancreatic prostanoid and pro-inflammatory cytokines in the WT mice. The KO mice also showed significant increases in pancreatic PGE$_2$, MCP-1, and MIP-2α. Additionally there was a significant reduction in all these mediators in the KO mice compared to the WT following caerulein stimulation with the exception of MIP-2α. Bars represent mean ± S.E.M. (n=5) * denotes significant difference between saline and caerulein treated mice of each mouse strain (p<0.05). # denotes significant difference between each mouse strain with the same treatment (p<0.05).

### 5.4.5 NFκB translocation in pancreatic acinar cells

The most prevalent activated form of NFκB is a heterodimer consisting of a p65 subunit and a p50 or p52 subunit, which contains transactivation domains necessary for gene induction (199). The active dimer translocates from the cytoplasm into the nucleus and activates transcription of targeted genes.

Both WT and CSE KO saline treated mouse had low basal NFκB activation in pancreatic acinar cells as
evidenced by p65 immunostaining in the nuclei, 1.5 ± 0.4 and 2.2 ± 0.4 nucleus per field of view respectively (Figure 5.7). Following caerulein treatment, the number of p65 positive nuclei significantly increased in both the WT and CSE KO mice, 14.4 ± 1.3 and 8.8 ± 1.0 respectively (p<0.05) (Figure 5.7). The caerulein treated CSE KO mice however had significantly less p65 positive nuclei in comparison to the corresponding WT mice (p<0.05) (Figure 5.7). This indicates a lower level of pancreatic acinar cell NFκB activation due to CSE deletion in response to pancreatitis.

![Figure 5.7 NFκB activation in pancreatic acinar cells.](image)

* denotes significant difference between saline and caerulein treated mice of each mouse strain.
5.4.6 Pancreatic acini response to caerulein stimulation

Caerulein hyperstimulation of WT pancreatic acini resulted in increased MCP-1 (1.45 ± 0.09 fold increase) and LTB₄ (1.47 ± 0.09 fold increase) release. MCP-1 and LTB₄ are mediators of the innate immune response by serving as potent chemoattractants to innate response cells (415, 416). Caerulein mediated release of MCP-1 by pancreatic acini has been previously reported in rat acini (291), however this is the first report that shows direct elicitation of LTB₄ release by hyperstimulated pancreatic acini. As with the in vivo findings (Figure 5.6b), CSE deletion resulted in attenuated MCP-1 secretion (1.45 ± 0.09 versus 1.18 ± 0.03 fold increase) by caerulein stimulated pancreatic acini and this was also observed with LTB₄ (1.47 ± 0.09 versus 1.23 ± 0.06 fold increase) (Figure 5.8).

**Figure 5.8** MCP-1 and LTB₄ release by WT and CSE KO pancreatic acinar cells in response to caerulein hyperstimulation. Freshly isolated pancreatic acini fragments from WT and CSE KO mice were treated with saline or caerulein (100 nM) for 1 h followed by detection of MCP-1 and LTB₄ in the supernatant. Caerulein treated pancreatic acini from WT mice showed a significant increase in MCP-1 and LTB₄ release over saline control. MCP-1 and LTB₄ release in caerulein treated CSE KO acini was significantly lower than the corresponding WT. Bars represent mean ± S.E.M. (n=3) * denotes significant difference between saline and caerulein treated mice of each mouse strain (p<0.05). # denotes significant difference between each mouse strain with the same treatment (p<0.05).

5.5 Discussion

This chapter describes the use of CSE KO mice in a model of acute pancreatitis to elucidate the role of CSE/endogenous hydrogen sulfide in inflammation. In this study, caerulein was used to induce
pancreatitis in CSE KO mice and their corresponding WT mice. Similar comparative experiments were carried out on freshly isolated pancreatic acini hyperstimulated with caerulein. From this we have shown that CSE KO mice have significantly less inflammation and subsequent tissue damage than their WT counterparts, suggesting that CSE facilitated production of hydrogen sulfide is a regulator of the immune system function and that inhibiting CSE may be useful in the clinical setting for treating auto-inflammatory conditions like pancreatitis.

5.5.1 CSE genetic deletion versus pharmacological inhibition

Currently, PAG is the most widely used inhibitor of endogenous hydrogen sulfide synthesis derived from CSE. It is a useful pharmacological inhibitor that has so far contributed to expanding our knowledge of the role of endogenous hydrogen sulfide under normal and pathological conditions. Studies using PAG have suggested that CSE is a major contributor towards increased circulating and tissue hydrogen sulfide as well as tissue hydrogen sulfide synthesizing activity in several models of inflammation (44, 45, 69, 189, 217). PAG inhibition of endogenous hydrogen sulfide synthesis have shown therapeutic effects in models of LPS induced endotoxaemia (44, 216), severe acute pancreatitis (66, 69), poly-microbial sepsis (189), burn-injury (45) and renal-injury (217, 218); however there have been studies in colitis (221), knee-joint synovitis (222) and liver-injury (223) that yielded contradicting results. However, the use of PAG as a specific CSE inhibitor has been implicated as a possible confounding factor due to its non-specific inhibition of other pyridoxal-5-phosphate dependent enzymes and the apparent pleiotropic effect of hydrogen sulfide could be partly due to this lack of specificity (discussed further in 1.5.4). This emphasises the importance of the present study, by using KO mice we have specifically targeted the CSE-hydrogen sulfide pathway. In doing so we have elucidated the direct effects of this pathway on pancreatitis without the possibility of the non-specific effects as seen with PAG based interventions.

A point to note however, is apart from just abolishing hydrogen sulfide synthesis derived from CSE, Ishii et al have also reported increased circulating cystathionine, homocysteine and methionine levels as well as a reduction in taurine levels in the knockout mice compared to the wild type (19). The levels of homocysteine were substantially higher but not at pathological levels reported in hyperhomocystenemia that is associated with a pro-inflammatory state (417-419) resulting in
development of atherosclerosis (419-421). Increase in circulating cystathionine and methionine have been reported in septic rats (422) and administration of cystathionine has been shown to be protective against liver and mucosal injury, presumably via increased synthesis and availability of cyst(e)ine and its antioxidative derivatives such as GSH (423, 424). However, in a CSE knockout mouse, the hypothesized beneficial antioxidative effect of GSH derived from cystathionine is not possible due to the inability to convert cystathionine to cysteine. Taurine, on the other hand, has been shown to be an anti-oxidant and play a protective role in tissue protection against oxidative damage (425, 426). Taken together, the protective effect of CSE deletion in pancreatitis is most probably not due to the reported derangement in circulating amino acids. Additionally, the absence of CSE did not substantially affect GSH levels as dietary supplementation of cysteine was adequate at keeping levels similar to that of wild types (427).

5.5.2 The role of CSE in pancreatic hydrogen sulfide synthesis and pancreatitis

CSE KO mice showed substantially less pancreatic hydrogen sulfide synthesising capacity compared to the WT mice (Figure 5.4). This would suggest CSE to be the major (but not the only) enzyme responsible for hydrogen sulfide synthesis in the pancreas. Interestingly, there does not seem to be functional compensation by other hydrogen sulfide synthesizing enzymes (CBS and 3MST) in the CSE KO mice pancreas to increase the hydrogen sulfide synthesizing activity to the levels measured in the WT mice. Furthermore, upon induction of acute pancreatitis with caerulein, while the WT mice showed higher levels of pancreatic hydrogen sulfide synthesizing enzyme activity as well as CSE expression, whereas there was no difference in hydrogen sulfide synthesizing enzyme activity between the saline and caerulein treated CSE KO mice (Figure 5.4). This confirms that CSE is the major enzyme responsible for the increase in pancreatic hydrogen sulfide synthesis in inflammation.

There was a significant protection against acute pancreatitis in CSE KO mice compared to the WT mice. In all parameters of pancreatitis analysed we found the KO mice were less severely affected by caerulein including hyperamylasemia, pancreatic MPO activity (an indicator of neutrophil infiltration) and pancreatic water content (an indicator of pancreatic edema) (Figure 5.1). As well as the histological analysis of pancreas sections (Figure 5.3) which showed significant reductions in acinar cell necrosis and oedema scores in the CSE KO mice compared to the WT mice.
5.5.3 Mechanisms of CSE/endogenous hydrogen sulfide regulation of the immune response in pancreatitis

5.5.3.1 CSE/endogenous hydrogen sulfide - substance P release (neurogenic inflammation)

Recent findings suggest possible mechanisms by which hydrogen sulfide may contribute to the inflammatory response observed during pancreatitis at the acinar cell level. It has been shown that caerulein hyper stimulation in acinar cells increases CSE expression as well as hydrogen sulfide synthesis in the tissue. Furthermore, inhibition of CSE with PAG significantly attenuate caerulein-induced upregulation of substance P and its receptor neurokinin-1 receptor (NK-1R) in pancreatic acini (304). Substance P is a known neurogenic inflammatory mediator that has been shown to play a deleterious role in acute pancreatitis (305). It has been shown to directly elicit acinar cell chemokine secretion (294), induce pancreatic microcirculatory dysfunction (428) and upregulate pancreatic cell adhesion molecule expression (429) in response to caerulein hyper stimulation, thus promoting leukocyte recruitment and contributing to the inflammatory response. Therefore inhibition of endogenous hydrogen sulfide synthesis in acinar cells could decrease the generation of substance P and subsequent leukocyte recruitment. Previous in-vivo findings have shown PAG to attenuate hydrogen sulfide and substance P synthesis in the pancreas, plasma and lungs of caerulein induced mice (244). In this study we have shown that CSE KO mice have lower MPO activity following acute pancreatitis indicating decreased leukocyte recruitment. These findings would further support the hypothesis of hydrogen sulfide acting as a positive upstream regulator of substance P expression thus contributing to the inflammatory response observed in pancreatitis.

5.5.3.2 CSE/endogenous hydrogen sulfide - NFκB activation

Increased acinar cell hydrogen sulfide synthesis has been shown to be associated with activation of NFκB (319, 360). In pancreatic acinar cells, this hydrogen sulfide mediated activation of NFκB has been thought to occur via activation of Src family kinase (SFKs) (319) and substance P mediated activation of TLR-4 receptor (360); while hydrogen sulfide has also been reported to directly sulfhydrate cysteine-38 of p65, enhancing its binding to the coactivator RPS3 resulting in its activation in macrophages (142). Pancreatic NFκB activation has been closely linked to the pathogenesis of pancreatitis; its activation
has been reported in multiple models of pancreatitis resulting in an upregulation of a plethora of pro-inflammatory cytokines, chemokines and cell adhesion molecules (reviewed in (430)). In this study we have shown a reduction of NFκB activation in the pancreatic acinar cells of CSE deficient mice in response to pancreatitis (Figure 5.7) as well as the reduction of NFκB dependent cytokines, IL-6, MCP-1 and MIP-2α (Figure 5.6). MCP-1 and MIP-2α are potent chemoattractants that are synthesized by pancreatic acinar cells in response to caerulein as well as substance P stimulation (294). Their reduction could account for the decreased leukocyte infiltration observed in the CSE KO mice of this study via the proposed hydrogen sulfide - substance P - NFκB pathway (319, 360). IL-6 however plays a more complex role in inflammation as it is thought to not only mediate the acute phase innate immune response but also direct the transition to an acquired response thus promoting resolution. However, pancreatitis is a non-pathogenic inflammatory event that resembles a more autoimmune like response in which case IL-6 is found to play a deleterious role (reviewed in (431)). Indeed high levels of serum IL-6 is strongly correlated with severe forms of pancreatitis that leads to greater morbidity and mortality (432) though the exact mechanism by which it exerts its effect is not clearly defined.

5.5.3.3 CSE/endogenous hydrogen sulfide - leukocyte recruitment

PGE2 is an eicosanoid that promotes vasodilation leading to edema as well as modulates immune response cells (323). Increased pancreatic and serum levels of PGE2 have been reported in pancreatitis (433-435) and their inhibition results in a protection against inflammation (434, 436). In this study, we have detected a significant reduction in pancreatic PGE2 level in the CSE KO mice compared to the WT in response to caerulein stimulation (Figure 5.6). This is in agreement with previous studies that show an hydrogen sulfide dependent increase in PGE2 and its metabolites in inflammatory models of sepsis (437) and hind-paw edema (438). This reduction could account for the observed reduction in edema and leukocyte infiltration. Furthermore, the concomitant decrease in MCP-1 could also be attributed to the recent evidence that shows PGE2 as a positive modulator of pancreatic acinar cell MCP-1 synthesis and secretion (439). Taken together, these findings suggest a possible new mechanism by which hydrogen sulfide may play a significant upstream role in the acinar cell inflammatory response
via modulation of prostanoid levels thus leading to increased chemokine production and subsequent recruitment of leukocytes.

MCP-1 and LTB₄ are potent chemoattractants that recruit innate response cells to a site of inflammation (415, 416). Here I have shown that 1 h caerulein stimulation resulted in significant release of MCP-1 and LTB₄ (~1.5 fold, Figure 5.8) in WT pancreatic acini. The release of these molecules could be one of the factors that contribute to the massive recruitment of inflammatory cells into the pancreas, a key feature of this disease. CSE gene deletion resulted in an almost complete attenuation of this observed response and suggests that CSE expression/endogenous hydrogen sulfide may play a key role in the progression of the pancreatitis associated inflammatory response at the acinar cell level.

A point to note here is that I was unable to detect changes in CSE expression levels in primary isolated pancreatic acini upon 1 h caerulein stimulation (refer to section 3.4.2). Therefore the observed increase in MCP-1 and LTB₄ release by wild-type pancreatic acini may not be due to increased hydrogen sulfide production via CSE upon caerulein stimulation. However, the decreased MCP-1 and LTB₄ release by CSE knockout pancreatic acini could be related to the pre-existing lower state of hydrogen sulfide exposure. The presence of basal CSE/endogenous hydrogen sulfide in the wild-type pancreatic acini could establish a basal ‘hydrogen sulfide modified environment’, i.e. in the form of s-sulfhydrated proteins, and in doing so prime these cells towards pathological or inflammatory cues.

5.5.3.4 Acute pancreatitis associated lung injury

Severe acute pancreatitis often results in the development of lung injury that closely resembles the acute respiratory distress syndrome (ARDS) associated with other processes such as shock, bacteraemia, ischemia/reperfusion, and burns (414). In this study, we found substantial protection in the lungs of CSE KO mice compared to the WT mice after 10 h of caerulein administration. There was significantly lower lung MPO activity and water content in the KO caerulein group compared to the WT group (Figure 5.5). Similar lung protection has been reported in mice treated with NK-1R receptor antagonist, CP-96345 (440) and NK1-R receptor knockout mice (305). Therefore the observed lung protection in this study could be the result of hydrogen sulfide acting as an upstream regulator of substance P expression in pancreatic acinar cells as suggested in previous studies (304).
In conclusion, the results presented in this chapter show that CSE/endogenous hydrogen sulfide plays a key pro-inflammatory role in caerulein induced pancreatitis and that the deletion of this gene results in significant protection against acute pancreatitis and associated lung injury. These findings were published (441) around the same time as two others (398, 442) and represent the initial reports using genetically deficient mice to determine the role of CSE in disease models of inflammation. This study also shows that CSE contributes to inflammation in acute pancreatitis at the acinar cell level through increased eicosanoid and chemokine synthesis possibly via NFκB activation which could contribute to the recruitment and activation of immune response cells. This would also imply that hydrogen sulfide, a product of CSE, could potentially be mediating these effects. However more work needs to be done to establish that these effects are indeed directly related to hydrogen sulfide.
6 Discussion and future work

The aims of this thesis were to investigate the dysregulation of CSE expression and its effect in the inflammatory response as well as to correlate these findings with changes in endogenous hydrogen sulfide levels. This was accomplished using macrophages in culture and a murine model of acute pancreatitis. My findings have shown upregulated CSE expression in pancreatic acini and macrophages in association with pancreatitis. These results also show that CSE contributed to the severity of the disease. However I was unable to correlate CSE expression with endogenous free hydrogen sulfide directly or indirectly as s-sulphhydrated proteins.

6.1 CSE expression in pancreatitis and the associated inflammatory response: a role for endogenous hydrogen sulfide

CSE dysregulation has been reported in various models of disease and injury (44, 45, 189, 217, 223, 325-330) but not in pancreatitis at the time this thesis was conceived and carried out apart from an in vitro study that showed increased pancreatic acini CSE expression in response to caerulein hyperstimulation (304). Here, I have shown that CSE is upregulated in the exocrine portion of the pancreas in acute pancreatitis along with increased pancreatic hydrogen sulfide synthesizing activity. These findings are corroborated by a recent report in rats using a more severe model of pancreatitis (69). However I was unable to detect increased CSE expression in caerulein hyperstimulated pancreatic acini in vitro and this could be due to experimental differences and limitations (discussed in section 2.4.5). Apart from the pancreas, I have also detected increased CSE expression in macrophages stimulated with plasma from pancreatitic mice. This result provides the only insight so far into macrophage-CSE expression in pancreatitis.

In spite of numerous studies pointing to CSE dysregulation in models of diseases (44, 45, 69, 189, 217, 223, 325, 327, 329, 330, 395, 398), there is very little evidence of this relating directly to changes in endogenous/tissue hydrogen sulfide levels (328, 394). Therefore the initial interest of this thesis was to develop a stronger correlation between observed dysregulation in tissue CSE expression with endogenous levels hydrogen sulfide in response to inflammation. However there were issues with developing a suitable high-throughput assay that would be specific enough as described in chapter 2.
An added layer of complexity is the interaction of hydrogen sulfide within the biological milieu; hydrogen sulfide is reported to have a high turnover rate in tissue homogenates with half-lives of between 2 to 6 mins depending on tissue type (33). This rapid disappearance of hydrogen sulfide has also been demonstrated in this thesis using liver homogenate (refer to 2.3.7). The disappearance of hydrogen sulfide was quicker under aerobic conditions due to oxidation resulting in formation of thiosulfate and sulfate (33). However, significant depletion was also observed under anaerobic conditions (32, 33) thus suggesting other means of hydrogen sulfide consumption/clearance. Two potential means of hydrogen sulfide consumption are through reduction of oxidised protein thiols (protein s-sulfhydration) (31, 344) or interaction with metal ion proteins (133, 134). A simple protein solution of 5% BSA (contains 2 free thiols per molecule and known to form intermolecular disulfide bonds) has been shown to rapidly ‘consume’ spiked hydrogen sulfide under anaerobic conditions thus further supporting the notion of interaction between hydrogen sulfide and proteins (32). This protein bound sulfane sulfur is also thought to be a store of endogenous hydrogen sulfide and has been shown to be released under reducing conditions in the brain (28). This highlights the complexity of estimating endogenous free hydrogen sulfide due to rapid turnover and contribution from labile sources; this also implies a current underestimation of endogenous hydrogen sulfide synthesising rate. As such, I had sought to use protein s-sulfhydration as an indirect measure of endogenous hydrogen sulfide levels. Another benefit of doing so was the potential of discovering possible hydrogen sulfide targets that are modified in the inflammatory response.

I elected to use a published biotin switch technique of labelling s-sulfhydrated proteins (31) as well as its variant, a biotin tag-switch technique that was reported to be more specific (344). Both methods involved an initial tagging/blocking of thiols and subsequent labelling of the intended target (protein persulfides with an R-S-SH group) with a biotin molecule (described in further detail in section 2.1.2). The proteins can then be separated on a western blot and readily probed for direct visualisation. This method provides a global overview of all proteins and was therefore an appealing choice since there is very little known about protein s-sulfhydration, particularly in the inflammatory context. However I was unsuccessful in replicating the protein s-sulfhydration assays based on previously published methods (31, 344) using the positive controls described, despite following the laboratory protocol sent by the authors.
There was substantial signal, however I was unable to detect changes in signal following treatment of tissue lysates with hydrogen sulfide (as a positive control) utilising these methods (Figure 2.9). Further investigation to try and decrease background signal by increasing MMTS concentration (R-SH blocker), decreasing protein ratio as well as varying SDS concentration to modulate protein denaturation (to expose free thiols) did not substantially alter or lower the background signal (Figure 8.7). I subsequently tried using another thiol blocker, NEM that has been reported to alkylate both R-SH as well as R-S-SH and is an irreversible method of blocking free thiols, again there was no change in background signal (Figure 8.7). Therefore the evidence points to a possible limitation in the protocol that prevents complete blocking of all endogenous free thiols (includes both R-SH as well as R-S-SH).

Another possibility could be the unfavourable redox state of the protein thiols or hydrogen sulfide donor (sodium sulfide). As described in 1.4.2, the sulfur atom from either the thiol group of a protein or hydrogen sulfide has to be in a more oxidised state relative to each other in order for a nucleophilic attack to occur resulting in the formation of a protein persulfide. Endogenous protein thiols can undergo oxidation by reactive oxygen species to form protein sulfenic acids, however basal levels are thought to be low as there are numerous mechanisms in place to effectively scavenge reactive oxygen species under non-stressed conditions (443, 444). Additionally, the protein sulfenic acids are transient and labile. Therefore it would be unlikely that the proteins thiols present in the homogenate of the assay would assume the role of the oxidant in the formation of protein persulfides, and if so would only constitute a small fraction of the total proteins present. Therefore if the protein thiols are predominantly present in the reduced state, the source of hydrogen sulfide should be in a more oxidised state for the protein persulfides to form. Sodium sulfide, the source of hydrogen sulfide, readily dissolves in solution to form H₂S as well as HS⁻, both of which possess sulfur in the -2 oxidation state which is identical to the oxidation state of sulfur in a reduced protein thiol (R-SH), therefore making the formation of protein persulfides impractical. However, Nagy et al have reported that the commonly used hydrogen sulfide donor, sodium hydrosulfide (NaHS), contains oxidised products such as polysulfides which have been suggested to be responsible for oxidation of protein thiols and in this case the formation of protein persulfides (74). Sodium sulfide is reported to be a purer form with some oxidation on the outer surface of the crystals that is removed by rinsing with water (34), which was the standard practice throughout this thesis. Although soluble hydrogen sulfide is susceptible to
oxidation by air, this is a slow process that can be catalysed by the presence of metal ions, however excess amounts of metal chelators were used in the s-sulfhydration experiments. Taken together, the choice of using hydrogen sulfide in its ‘pure’ form could have been a possible reason for not observing protein persulfide formation in this thesis. However, this does not mean that endogenous protein persulfide formation is not possible, particularly under inflammatory conditions, as large amounts of reactive oxygen species are generated leading to oxidative stress and significant increase in protein sulfenic acid formation (443-445).

Having not been able to measure changes in endogenous hydrogen sulfide or protein persulfide levels, I will discuss the implications of CSE upregulation on hydrogen sulfide production in the pancreas and macrophages in response to pancreatitis in the following sections. CSE is classically known for its canonical role in the transsulfuration pathway for the conversion of cystathionine to cysteine, α-ketobutyrate and ammonia. This also represents the sole pathway for endogenous synthesis of cysteine; an amino acid required for protein synthesis and the production of other essential molecules that include GSH, coenzyme A, taurine, and inorganic sulfur (446). Apart from cysteine synthesis, CSE is also capable of generating hydrogen sulfide as a by-product through cysteine-dependent β and homocysteine-dependent γ-reactions (21). Among them, the α,β-elimination of cysteine accounts for ~70% of hydrogen sulfide synthesis under physiological concentrations of cysteine (100 μM) and homocysteine (10 μM) (21). Cysteine is the direct substrate for hydrogen sulfide synthesis via CSE as well as the precursor to GSH and taurine synthesis through additional enzymes. This creates a competition between GSH, taurine and hydrogen sulfide synthesis from cysteine and the balance between substrate-enzyme-product levels determines which molecule(s) is/are made at any one point (447). Figure 6.1 illustrates the CSE mediated pathways and products.
Figure 6.1 Products of CSE mediated pathways. The canonical role of CSE is through the transsulfuration pathway leading to endogenous cysteine synthesis. Cysteine is used as a substrate GSH synthesis and excess cysteine converted to hypotaurine and taurine, a less toxic product. GSH and taurine possess antioxidant properties. Cysteine also functions as a substrate for CSE to yield hydrogen sulfide via an alternative pathway. Hydrogen sulfide has been shown to have both pro and anti-inflammatory effects. The balance and shift between CSE mediated synthesis of taurine, GSH and hydrogen sulfide depends on the levels of substrate, enzyme and product at any one point (447). (1) Betaine homocysteine methyltransferase (2) Methionine adenosyl transferase (3) Methyl transferase (4) Adenosyl homocysteinase (5) Cystathioninone $\beta$-synthase (6) Cysteine dioxygenase (7) Aspartate aminotransferase (8) Cysteinesulfinate decarboxylase (9) unknown (10) $\gamma$-Glutamyl cysteine synthetase (11) GSH synthetase.

6.1.1 Pancreatitis induced CSE upregulation in the pancreas

The results of this thesis have shown the capacity for substantial hydrogen sulfide production in wild type pancreatic homogenate (17.4 nmol / mg protein/ h) which was increased (~1.3 fold) upon induction of acute pancreatitis (refer to 3.4.1). In pancreatitis, there is a diminished level of pancreatic and circulating cysteine (448) and circulating cysteine (449). This is concomitant with a lowering of GSH levels too probably due to increased oxidative stress (450). GSH depletion has been shown to increase CSE expression thus
promoting flux through the transsulfuration pathway to increase GSH synthesis (451). This could be a reason for the observed upregulation in pancreatic exocrine CSE expression shown in this thesis. This would also suggest that the availability of cysteine for hydrogen sulfide synthesis via CSE could be limited as it is redirected to support antioxidative mechanisms via GSH and taurine synthesis. However it is interesting to note that prevention of cysteine depletion in rats with pancreatitis did not reverse GSH loss (448) which could suggest that the impaired GSH production is independent of cysteine levels in pancreatitis and the balance of pancreatic cysteine consumption could therefore shift to either taurine or hydrogen sulfide synthesis. However it is difficult to speculate as there are currently no reports of pancreatic taurine or hydrogen sulfide levels (due to the difficulty in detecting free hydrogen sulfide as previously described in chapter 2) in response to pancreatitis. It is unclear at this juncture as to the direct consequence of increased pancreatic exocrine CSE expression in pancreatitis and if it would serve to alter endogenous taurine, GSH or hydrogen sulfide synthesis. However, the significantly impaired pancreatic hydrogen sulfide synthesising ability in CSE knockout mice (refer to section 5.4.2) suggests there could be an effect of basal pancreatic hydrogen sulfide levels that would influence the ability of the tissue/cells to respond to inflammatory stimuli. For example, I have shown that primary isolated pancreatic acini did not upregulate CSE expression upon 1 h caerulein stimulation; however, acini lacking the CSE gene had impaired inflammatory response as shown by decreases in chemokine and eicosanoid release (refer to section 5.4.6). However, the decreased inflammatory response observed in CSE knockout pancreatic acini could be related to the pre-existing lower state of hydrogen sulfide exposure. The presence of basal CSE/endogenous hydrogen sulfide in the wild-type pancreatic acini could establish a basal ‘hydrogen sulfide modified environment’, i.e. in the form of s-sulfhydrated proteins, and in doing so prime these cells towards pathological or inflammatory cues.

6.1.2 Pancreatitis induced CSE upregulation in the macrophage

Currently, there is only one report that shows macrophage hydrogen sulfide synthesizing ability in real-time using a miniaturized hydrogen sulfide micro-respiration sensor under saturating levels of L-cysteine and PLP and this is increased upon LPS stimulation (55). Macrophages possess the plasma membrane cystine transporters, System xc’, therefore enabling the import of extracellular cystine which is readily reduced to cysteine under the reducing intracellular environment (452). Plasma
contains substantial levels of cysteine (100 – 200 μM) and activated macrophages have been shown
to actively import cystine and release almost equivalent amounts of cysteine (453-455). This large
influx of cysteine is partly utilised for GSH synthesis though there have been no reports of intracellular
taurine synthesis although macrophages have been shown to import taurine via taurine transporters
instead (426). Therefore we could infer that there is a readily available pool of intracellular cysteine in
macrophages independent of CSE mediated cysteine synthesis via the transulfuration pathway. In fact
the increase in CSE expression and high intracellular cysteine levels following macrophage activation
is ideal for hydrogen sulfide synthesis because there is not a need for intracellular cysteine synthesis
via CSE and cysteine is the preferred substrate for endogenous hydrogen sulfide synthesis via α,β-
elimination by CSE (21), particularly due to the lack of CBS expression in macrophages (refer to section
4.4.2). Taken together, this suggests that the upregulation of CSE in activated macrophages would
most likely result in the production of hydrogen sulfide and the elicitation of its effects. This also
highlights the relevance of CSE/endogenous hydrogen sulfide to the inflammatory response as
macrophages are an important component of the innate immune cells that drive the inflammatory
response.

6.2 Role of CSE in acute pancreatitis and associated inflammatory response

6.2.1 The effect of CSE gene deletion on the severity of acute pancreatitis

In order to determine the role of CSE in an inflammatory response, I have compared the effect of CSE
gene deletion in mice using acute pancreatitis as the disease model. Mice genetically deficient in CSE
displayed normal physical characteristics when fed standard laboratory chow containing L-cysteine
(16). These mice developed pancreatitis upon induction with caerulein showing typical signs of the
disease such as elevated plasma amylase levels (a measure of pancreatic dysfunction), increased
pancreatic and lung MPO activity (a measure of neutrophil infiltration), as well as acinar cell necrosis
and pancreatic oedema (a measure of pancreatic injury) (refer to sections 5.4.1 and 5.4.3). However
the magnitude of disease severity was significantly lower when compared to wild type (WT) control
mice using the parameters described.
These results inconvertibly show that CSE plays a pro-inflammatory role in acute pancreatitis. Earlier studies (66, 69, 318), using PAG as a pharmacological inhibitor, have indicated that hydrogen sulfide synthesised by CSE acts as a pro-inflammatory mediator. However a key contribution of the present findings is the use of genetic deletion as opposed to PAG which is known to inhibit other pyridoxal-5-phosphate dependent enzymes that could directly or indirectly affect the inflammatory response (reviewed in (243). A point to note is that apart from just abolishing hydrogen sulfide synthesis derived from CSE, substantial increase in circulating cystathionine and homocysteine levels as well as a decrease in taurine levels were observed in knockout mice compared to wild type (19). Nonetheless, the protective effect of CSE deletion in acute pancreatitis is most probably not due to the reported derangement in circulating amino acids; the levels of homocysteine were not at pathological levels reported in hyperhomocystenemia (417-419), the hypothesized beneficial antioxidative effect of GSH derived from cystathionine (423, 424) is not possible due to the inability to convert cystathionine to cysteine, taurine, on the other hand, has been shown to be an anti-oxidant and play a protective role in tissue protection against oxidative damage (425, 426) (discussed in section 5.5).

These findings were published (441) around the same time as two others (398, 442) and represent the initial reports using genetically deficient mice to determine the role of CSE in disease models of inflammation. The other two reports (398, 442) used CSE deficient mice that were developed by Yang et al 2008 (237). Zhang et al 2013 used a model of ovalbumin (OVA)-induced acute asthma and showed aggravated airway hyperresponsiveness (AHR), increased airway inflammation, and elevated levels of Th2 cytokines in bronchoalveolar lavage fluid after OVA challenge in the CSE deficient mice (398). This corroborates with a previous finding that exogenous hydrogen sulfide mitigates the severity of asthma (397); however there has not been any prior studies using PAG as a CSE inhibitor in animal experiments of asthma to compare with. The second report has shown CSE to protect against renal ischemia-reperfusion injury presumably by modulating oxidative stress through the production of hydrogen sulfide.(442). Again this corroborates with previous findings using a similar ischaemia-reperfusion injury model of the kidney with exogenous hydrogen sulfide (228, 247) as well as PAG (124). However there are two reports that show a detrimental effect of CSE using gentamicin (218) and cisplatin (217) as an inducer of renal injury. Taken together, these gene deletion and pharmacological inhibition
studies suggest that CSE does play a role in the inflammatory response; however, the effect of CSE inhibition/deletion could vary with the method of disease induction and the targeted organ of damage.

6.2.2 The effect of CSE gene deletion on the inflammatory response in acute pancreatitis: possible roles for endogenous hydrogen sulfide

An interesting feature of acute pancreatitis is the massive infiltration of leukocytes into the pancreas stemming from an uncontrolled immune response. This unleashes the arsenal of tissue destructive machinery from the innate immune response cells leading to extensive pancreatic damage and if not contained, would result in distant organ damage. Chemokines and leukotrienes are key molecules that serve as potent chemoattractants as well activators of leukocytes (416, 456). These molecules are synthesised and released in large quantities by activated leukocytes as a means of propagating the inflammatory response and has been shown to be elevated in the pancreas during pancreatitis (435, 457, 458). Although activated leukocytes are the predominant source of chemokines and leukotrienes in an inflammatory response, pancreatic acinar cells have also been shown to release chemokines MCP-1 and MIP-2α upon caerulein stimulation in vitro (291, 294) though there are no reports on leukotriene release as yet. Here, I have shown that stimulation of pancreatic acini with 100 nM caerulein for 1 h elicited MCP-1 as well as LTB₄ release (refer to section 5.4.6) which could contribute to the recruitment and activation of resident and peritoneal macrophages thus initiating the inflammatory response. The caerulein induced release of these molecules was substantially reduced with CSE gene deletion (refer to section 5.4.4); this could possibly explain the observed decrease in pancreatic leukocyte infiltration during pancreatitis thus reducing the extent of pancreatic damage and severity of the disease. Attenuation of other classical pro-inflammatory mediators such as the cytokine, IL-6 and prostanoid, PGE₂ were also observed in the pancreas with CSE gene deletion (refer to section 5.4.4) and this could be a consequence of lessened disease severity due to lowered pancreatic leukocyte recruitment.

However, a recent report has shown PGE₂ as a positive modulator of pancreatic acinar cell MCP-1 synthesis and secretion (439) thus suggesting that CSE modulation of eicosanoids could be an upstream event leading to increased and subsequent MCP-1 synthesis and release in pancreatic acinar cells. The initial step in PGE₂ and eicosanoid synthesis involves the hydrolysis of membrane glycerophospholipids
to arachidonic acid, cytosolic phosholipase A\(_2\) (cPLA\(_2\)) is the main enzyme responsible for this initiating step and its activation is a major controlling point in the synthesis of eicosanoids (459, 460). cPLA\(_2\) requires Ca\(^{2+}\) binding for activation (459), endogenously synthesised hydrogen sulfide has been shown to increase intracellular Ca\(^{2+}\) levels in pancreatic acinar cells in a concentration dependent manner (320). Taken together, the ability for endogenous hydrogen sulfide to modulate pancreatic acini intracellular Ca\(^{2+}\) levels offers a possible mechanism for CSE mediated increase in pancreatic MCP-1 synthesis via PGE\(_2\).

Substance P is a known neurogenic inflammatory mediator that has been shown to play a deleterious role in acute pancreatitis (305). The pancreas is innervated with substance P containing peripheral nerves (461, 462) and the release of substance P from these nerve endings is mediated by extracellular calcium influx (463) via ion channels (464, 465). Hydrogen sulfide has been shown to activate a variety of ion channels (reviewed in (466)) and among them the nociceptive ion channels TRPA1 (105, 112, 113) and TRPV1 (114) (79, 198) which are known to induce substance P release (462, 467-469). Previous in-vivo and in vitro findings have shown PAG to reduce hydrogen sulfide and substance P synthesis in the pancreas and isolated pancreatic acini of caerulein induced pancreatic mice (244) and cells (304) respectively. Taken together, this would suggest another possible mechanism by which CSE could contribute to the inflammatory response via hydrogen sulfide activation of ion channels on peripheral neuron endings resulting in release of substance P thus causing neurogenic inflammation. Substance P has been shown to stimulate chemokine release in pancreatic acinar cells (294) such as MCP-1 and MIP-2\(\alpha\) which correlates with findings in this thesis.

NF\(\kappa\)B represents a family of transcription factors that plays an important role in inflammation by promoting the transcription of pro-inflammatory genes (199). Pancreatic NF\(\kappa\)B activation has been closely linked to the pathogenesis of pancreatitis and its activation has been reported in multiple models of pancreatitis (reviewed in (430)). Substance P has been shown to activate NF\(\kappa\)B in pancreatic acinar cells (294, 470). Here I have shown that CSE gene deletion results in reduced NF\(\kappa\)B activation in pancreatic acinar cells in acute pancreatitis (refer to section 5.4.5). Taken together, this would suggest a CSE-substance P-NF\(\kappa\)B dependent pathway as a mechanism by which endogenous hydrogen sulfide could contribute to the pathogenesis and inflammatory response of acute pancreatitis via NF\(\kappa\)B mediated upregulation of pancreatic MCP-1, MIP-2\(\alpha\) and IL-6 synthesis.
High mobility group box 1 (HMGB1) is a nuclear protein and a component of DAMPs with extracellular inflammatory cytokine activity (471), thus making it a crucial mediator of the response to infection, injury and inflammation (472). HMGB1 is sensed by endothelial cells and monocytes/macrophages via TLR2, TLR4 and receptor for advanced glycation endproducts (RAGE) receptors resulting in activation of NFκB and the increased synthesis and release or pro-inflammatory mediators such as cytokine, chemokines and adhesion molecules (471). Increased serum, pancreatic and lung levels of HMGB1 have been reported in animal models of acute pancreatitis and there is a positive correlation of serum HMGB1 levels and disease severity (reviewed by (473)). The inhibition of HMGB1 expression and neutralisation of extracellular HMGB1 in acute pancreatitis resulted in marked decrease of pancreatic and circulating cytokine and chemokine levels as well as amelioration of the disease (474-476); this suggests a pivotal role of HMGB1 as a driver of the uncontrolled inflammatory response in acute pancreatitis. The redox state of HMGB1 cysteine 106 residue modulates its activity; a reduced thiol state is required for HMGB1 to induce nuclear NFκB translocation in macrophages and oxidisation results in inhibition of activity (477). It has been hypothesised that hydrogen sulfide could function as a reducing agent thus keeping the HMGB1 cysteine 106 thiol in the reduced state and therefore maintaining its pro-inflammatory effect (478). This is another mechanism by which hydrogen sulfide may function as a pro-inflammatory mediator, particularly in sterile inflammatory conditions where extensive tissue damage occur thus releasing DAMPs such as HMGB1.

Figure 6.2 summarises the proposed mechanism by which endogenous hydrogen sulfide could contribute to the enhanced pancreatic cytokine, chemokine and eicosanoid synthesis in acute pancreatitis.
Figure 6.2 Proposed mechanisms of hydrogen sulfide mediated increase in pancreatic pro-inflammatory mediators in pancreatitis. (A) Activation of ion channels on pancreatic acinar cells leading to intracellular Ca$^{2+}$ increase > activation of PLA$_2$ > synthesis of arachidonic acid, precursor for PGE$_2$ and LTB$_4$ > potentiation of MCP-1 transcription by PGE$_2$. (B) Activation of ion channels on nerve endings in the pancreas leading to intracellular Ca$^{2+}$ increase > stimulation of substance P release > activation of NK-1 receptors on acini > neurogenic inflammation – transcription of chemokines via NFκB, increase in microvascular permeability, oedema. (C) Reduction of oxidised HMGB1 on the cysteine 106 thiol group thus maintaining its active pro-inflammatory effect on surrounding endothelial cells and macrophages; activation of NFκB and transcription of cytokines and chemokines.

6.3 Summary of findings

My hypotheses were that CSE contributes to the inflammatory response in acute pancreatitis by increasing endogenous hydrogen sulfide synthesis and genetic deletion of CSE would lessen the severity of acute pancreatitis and the associated inflammatory response. The overall aim was to validate previous reports on the inflammatory role of CSE/ endogenous hydrogen sulfide in acute pancreatitis by means of genetic deletion instead of pharmacological inhibition and to investigate the relevance of CSE expression in the immune response. The specific aims are shown below with a brief summary of findings following:
1) To refine an existing simple high-throughput method that would fulfil the criteria for specific and sensitive detection of free hydrogen sulfide in tissue with standard laboratory equipment.

A method of measuring free hydrogen sulfide was established based on the spectrophotometric Methylene Blue method of detection that circumvented the issue of acid labile sulfide release and with improved sensitivity by concentrating precipitated hydrogen sulfide. This method should in principle be specific towards measuring only free tissue hydrogen sulfide with the added benefits of the simple protocol, level of high-throughput and the requirement of a standard spectrophotometer for detection. However subsequent measurement of free hydrogen sulfide in murine tissue yielded higher than expected values in comparison to currently perceived best method of detection by gas chromatography (37). This could be due to endogenous labile sulfide release due to strong alkaline conditions of the assay.

2) To correlate CSE dysregulation with changes in hydrogen sulfide mediated protein modifications (s-sulfhydrated proteins) as an indicator or footprint of CSE mediated hydrogen sulfide synthesis.

I was unable to test this correlation due to inability in replicating published methods of proteins s-sulfhydration detection using the biotin switch assay (31) and a variant, biotin tag-switch assay (344) despite various attempts to optimise the conditions of the assays. A recent report by Nagy et al (74) suggested that the formation of s-sulfhydrated proteins by treatment with hydrogen sulfide is due to its oxidised form present in hydrogen sulfide donors as this would result in a favourable nucleophilic substitution reaction. This concept was not explored in this thesis and could be a reason for the inability to replicate the published methods of proteins s-sulfhydration detection. Additionally, sodium sulfide (Na$_2$S), the source of hydrogen sulfide, was routinely rinsed prior to use to remove ‘oxidised hydrogen sulfide contaminants’ as suggested in a previous report (34) in order to obtain a pure source of hydrogen sulfide as possible. This could unwittingly support the hypothesis postulated by Nagy et al.

3) To determine changes in CSE expression in the pancreas and macrophages in response to acute pancreatitis, the molecular mechanisms involved.

I have shown that pancreatic CSE expression is upregulated by western blotting (~33 %) following caerulein induced acute pancreatitis in mice and this is supported by immunohistochemistry that
shows increased staining in the exocrine portion of the pancreas. These results are supported by a recent publication that used a sodium taurocholate model of pancreatitis in rats (69). Taken together, these findings affirm the upregulation of pancreatic CSE in response to pancreatitis and this occurs in the pancreatic acinar cells. In contrast to the in vivo findings, there was no detectable change in in vitro caerulein stimulated pancreatic acini CSE expression by qPCR and western blotting. This discrepancy could be due to limitations of the in vitro system that does not include the complex interaction of pancreatic acinar cells with other components of the inflammatory response such as resident tissue macrophages and circulating neutrophils. Another factor could be due to the shorter induction period used in the in vitro system.

Increased expression of CSE in macrophages have also been shown under inflammatory conditions of acute pancreatitis (~2 fold), this occurs via a PI3K-sp1 dependent pathway. These results represent the only report that links macrophage CSE upregulation to an in vivo model of inflammatory disease. This shows that CSE/endogenous hydrogen sulfide may play a role in macrophage function and therefore the inflammatory response at large.

4) To determine the effect of CSE on the inflammatory response and disease severity using mice genetically deficient in CSE as an alternative to pharmacological inhibition.

I have shown that CSE plays a key pro-inflammatory role in caerulein induced pancreatitis and that the deletion of this gene results in significant protection against acute pancreatitis and associated lung injury. These findings were published (441) around the same time as two others (398, 442) and represent the initial reports using genetically deficient mice to determine the role of CSE in disease models of inflammation. This study shows that CSE contributes to inflammation in acute pancreatitis at the acinar cell level through increased eicosanoid and chemokine synthesis possibly via NFκB activation which could contribute to the recruitment and activation of immune response cells. These CSE gene deletion studies serve as confirmation to previous reports of CSE playing a role in the inflammatory response without the possible confounding non-specific effects of PAG.

A summary of these findings are shown in Figure 6.3
Figure 6.3 Advancement of knowledge from this thesis. The results of this thesis have shown (1) Upregulation of pancreatic and macrophage CSE expression in response to acute pancreatitis (2) CSE gene deletion to decrease the severity of acute pancreatitis in mice (3) The pathway leading to upregulated CSE expression in LPS and pancreatic plasma stimulated macrophages.

6.4 Future work

Although the field of hydrogen sulfide has grown exponentially, there are some fundamental aspects that appear to be lagging. More specifically the lack of correlation between CSE dysregulation with changes in endogenous hydrogen sulfide levels. This lack of correlation casts doubt as to whether the observed effects of CSE dysregulation are actually related to its hydrogen sulfide synthesising ability or due to other downstream CSE mediated roles. However the lack of this evidence is understandable due to the difficulty in measuring endogenous hydrogen sulfide (discussed in chapter 2). Another aspect that should be focused on is the elucidation of the mechanisms by which hydrogen sulfide exerts its observed physiological effects. So far, hydrogen sulfide has been reported to function as an antioxidant (96, 97, 99, 135) (165) through s-sulfhydration of proteins (102, 141-146) and via interaction with metal ion containing proteins (133, 158-163, 168). However there are still very few reports of the occurrence of these events as well as the elucidation of its implications in in vivo models
of disease and inflammation. Therefore, future work should be focused on attempting to more accurately measure changes in endogenous hydrogen sulfide levels in in vivo models of disease and inflammation particularly in tissues where CSE dysregulation is observed as well as determining the mechanisms by which hydrogen sulfide may exert its effect.

6.4.1 Measuring endogenous hydrogen sulfide

Currently the monobromobimane-HPLC method of hydrogen sulfide detection offers a good balance in specificity, sensitivity and level of throughput (36). However the method has been established to only detect free hydrogen sulfide in biological solutions such as plasma (26, 46) and therefore needs to be adapted for tissues. This method could also be coupled with mass spectrometry to increase the specificity of the assay. Once established, this method could be used to measure endogenous hydrogen sulfide changes in plasma and the pancreas over the course acute pancreatitis and if so, at what point of the disease would it be relevant to. Additionally, it would also be worthwhile to measure hydrogen sulfide levels in the lymphatic system, particularly via the thoracic duct, as this is the point where the peritoneal fluid is carried into the systemic circulation via the subclavian vein. This could be achieved by cannulation of the thoracic duct. Any measured changes in endogenous hydrogen sulfide levels could then be correlated to changes in CSE expression to establish a relationship between the enzyme and product. While utilising the monobromobimane-HPLC method would provide an estimation of the end-point state of endogenous free hydrogen sulfide levels, this may not represent the true potential amount hydrogen sulfide being made as it is in a state of constant flux. Therefore a complimentary real-time in situ method of detection could provide a better appreciation of the amount of endogenous hydrogen sulfide generated.

This could be achieved via the use of fluorescent probes coupled with real-time imaging. A particularly appealing choice of fluorescent probe would be one designed by Chen et al (2014) based on a modified form of hsGFP (hydrogen sulfide sensitive green fluorescence protein) that incorporates an unnatural amino acid (p-azidophenylalanine) (58). This renders the GFP sensitive to reduction by hydrogen sulfide specifically resulting in increased fluorescence intensity. The added benefit of using this system is the ability to transfect the hsGFP gene thus getting the cell of interest to generate its own hydrogen sulfide probe, a stable transfection and establishment of an hsGFP cell line is also possible. The hsGFP
system would however be limited to in vitro cell culture studies which would sit well with the macrophage studies. Macrophages reside in numerous tissues and are the first line of sensors, initiators and modulators of the immune response. It has already been established that macrophages have low basal CSE expression and is upregulated upon stimulation with inflammatory stimuli. Additionally, there is no detectable expression of CBS. Therefore this could provide a relatively ‘clean’ system to detect changes in CSE mediated endogenous hydrogen sulfide synthesis and identification of its targets in response to inflammation.

6.4.2 Detecting/measuring protein persulfides

Protein s-sulfhydration or formation of protein persulfides via interaction of hydrogen sulfide derivatives and thiol groups of proteins offer a promising mechanism by which hydrogen sulfide may directly exert its effects. Although the direct reaction between hydrogen sulfide in its reduced form (H\_2S and HS\^-) and a protein thiol (R-SH) is not feasible, the inflammatory state is associated with generation of reactive oxygen species. These species could in turn oxidise either hydrogen sulfide or the thiol group of a protein thus making it favourable for both molecules to react and form protein persulfides. Therefore, future experiments testing protein s-sulfhydration would incorporate the use of oxidised hydrogen sulfide or protein thiol species to facilitate protein persulfide formation. Follow up experiments to test the physiological relevance of these oxidised species could be conducted with both reduced protein thiols and hydrogen sulfide in the presence of known endogenous reactive oxygen species to determine if the endogenous formation of protein persulfides could indeed be feasible. Apart from the biotin switch assay, an alternative method of simultaneous fluorescent labelling of protein thiols (R-SH) and protein persulfides (R-S-SH) could be used. This can be achieved by first using a known fluorescently tagged alkylating reagent (ie maleamide) that would tag all thiol species (R-SH and R-S-SH). This would be followed by a reduction step that would only cleave the previously alkylated protein persulfides (R-S-SH) to form a simple thiol (R-SH). There would then be a second round of alkylation with a different fluorescent label and this should in theory alkylate/tag the reduced protein persulfides. Finally, the samples can be resolved by western blot and visualised on a molecular imager. Ideally the two fluorescent tags chosen should be well distinguishable such as near infrared tags. The benefit of using this alternative fluorescent method is the ability to observe both
thiol and s-sulphhydrated states of the protein simultaneously which could provide a frame of reference for the degree of s-sulphhydration.

While the biotin switch and fluorescent assays would offer a broad view of s-sulphhydration state in a complex mixture of proteins such as cell or tissue lysates, it would also be beneficial to employ a separate technique that is more sensitive, specific and quantitative to confirm these results. This would provide inconvertible evidence of protein s-sulphhydration (formation of protein persulphides). Mass spectrometry would satisfy all these criteria but would require a purified source of protein in order to function reliably. Therefore this could be a complimentary method to study specific proteins that are key to the inflammatory response such as NFκB and HMGB1 among others. Here, an antibody specific pull down method could also be employed to isolate certain proteins of interest from cell or tissue lysates in order to be analysed by mass spectrometry. This analysis could be performed on samples from activated or non-activated macrophages as well as non-inflamed and inflamed tissues to compare levels of s-sulphhydration.

6.4.3 Clinical relevance of CSE/ endogenous hydrogen sulfide in acute pancreatitis

Another future direction would be to investigate CSE/ endogenous hydrogen sulfide in the context of clinical acute pancreatitis. The results from this thesis offers a promising avenue of research in the understanding of acute pancreatitis pathophysiology and these findings should be investigated in a clinical setting to establish its relevance. Needle aspirates of pancreas could be obtained to test for changes in CSE expression, endogenous hydrogen sulfide and protein persulphide levels. Since patients would generally arrive when the disease has significantly progressed, it would be unclear at that stage if the prevailing disease would be in either the SIRS, CARS or MARS state. Therefore samples could be taken over the course of recovery as well as a period after to track changes over time and to establish a baseline. These samples could also be assayed for pro and anti-inflammatory cytokines in order to discern what state of inflammatory response the patient is at. This would help establish a correlation between CSE/ endogenous hydrogen sulfide and the inflammatory process thus furthering our knowledge on its role in the inflammatory response. In instances where peritoneal drainage or lavage is conducted, these samples could also be used to measure levels of hydrogen sulfide and protein.
persulfides. In addition, inflammatory cells such as neutrophils and macrophages could also be isolated from the peritoneal fluid to measure CSE expression and protein persulfide levels. This would provide a broader understanding of CSE/ endogenous hydrogen sulfide not only in terms of the pancreatic response in acute pancreatitis but the inflammatory response at large.

6.4.4 Role of CSE/ endogenous hydrogen sulfide in other inflammatory diseases and assessing the therapeutic potential?

In order to further establish the role of CSE in the inflammatory response, it would be useful to conduct more pre-clinical studies in other models of inflammatory diseases using mice genetically deficient in CSE. This would aid in affirming previous reports using PAG without the confounding effects of its non-specific activity. Ideally, a variety of diseases with differing aetiologies should be employed in order to help differentiate the possible role of CSE/ endogenous hydrogen sulfide in the development of the disease as opposed to the associated inflammatory response. In order to effectively compare the effect of CSE gene deletion across these various diseases, there needs to be a common set of inflammatory outcomes deemed to be relevant to the inflammatory response and pertinent to each disease. These could include leukocyte activation and infiltration, cytokine and eicosanoid production as well as vascular permeability.

While the use of CSE deficient mice is useful in establishing its effect, its therapeutic potential would be questionable as this deficiency is present prior and during the early phase of the disease. Therefore, in order to determine if there could be potential therapeutic value in modulating CSE activity, a separate set of experiments involving specific and time dependent inhibition of CSE would be essential. Initial experiments could utilise conditional knockout mice which would be more economically viable though not clinically relevant yet. This would allow targeted CSE inhibition studies at specific periods during the course of the disease where it would be more clinically relevant. The effect of CSE inhibition at these different stages may yield different outcomes because immunopathogical diseases may comprise of different states of SIR, CARS and a mix of both, MARS. If the outcome was promising, these experiments could progress towards the use of small interfering RNA (siRNA) which would be as specific but costly. The benefit of siRNA technology is that it can be administered as a drug and has the potential of being targeted towards specific organs and even immune cells.
Figure 6.4 summarises the current knowledge and suggested future work. Answering these questions would better establish the presence and relevance of CSE and endogenous hydrogen sulfide as well as greatly further our understanding of their role in the inflammatory response.

**Figure 6.4 Proposed future work.** This figure illustrates the current body of knowledge on pancreatic and macrophage CSE dysregulation as well as the effect of CSE inhibition in acute pancreatitis. The evidence from this thesis warrants subsequent investigation of CSE/endogenous hydrogen sulfide in clinical acute pancreatitis. It also highlights future work to correlate these changes in CSE expression with endogenous hydrogen sulfide levels and to determine the possible direct targets of hydrogen sulfide. Additionally, CSE deficient mice could be used in other animal models of disease and inflammation to confirm previous findings of CSE effect using PAG.
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8 Appendix

8.1 Assessing specificity of western blot CSE antibody

Figure 8.1 Assessing specificity of primary antibody used to detect CSE protein by western blotting. Mouse monoclonal primary antibody towards CSE (Abnova, Taiwan) was paired with a goat anti mouse HRP conjugated secondary antibody (Pierce biotechnology, USA). Figure shows a western blot performed on mouse pancreatic lysates resolved on a 10% polyacrylamide gel and transferred onto a nitrocellulose membrane. The 'WT' lane represents lysate prepared from a C57BL6 wild type mouse, 'KO' lane represents lysate prepared from a CSE knockout mouse and lane 'M' represents the molecular weight marker that has been underlayed. The expected molecular weight of CSE is ~ 43 kDa which corresponds to the strong signal observed between the 37 and 50 kDa range in the WT lane but absent in the KO lane.
8.2 Confirming specificity of CSE and β-actin PCR primers

**Figure 8.2 Assessing specificity of β-actin and CSE PCR primers control.** Primers were designed using NCBI Primer blast to recognise and amplify mouse mRNA of β-actin and CSE. The predicted amplicon sizes were 128bp and 115bp for β-actin and CSE respectively. This figure represents a run of PCR reaction products performed using total RNA extracted from wildtype (WT) and CSE knockout (KO) mice liver on a 1.7% agarose gel. RNA was extracted using TRIZOL and reverse transcribed using Oligo(dT)20 as the primer. PCR was performed using primers described in Table 5. Sequencing of β-actin and CSE bands showed alignment with the predicted amplicon sequence.
Figure 8.3 qPCR products resolved on agarose gel. At the end of the qPCR run (40 cycles), products were resolved on a 1.7% agarose gel to determine if amplification was specific. This was confirmed by the presence of one distinct band for each lane for both β-actin and CSE.

8.3 Optimisation of CSE immunostaining
Figure 8.4 No primary antibody control. Immunostaining of pancreas without the CSE primary antibody and counterstained with hematoxylin. Immunostaining was performed with a rabbit specific Horse Radish Peroxidase (HRP) /3,3’-Diaminobenzidine (DAB) Avidin-Biotin Complex (ABC) Detection IHC Kit by Abcam, Cambridge UK as described in section 3.3.6. There was no detectable background CSE staining in the absence the primary antibody.
Figure 8.5 Testing primary antibody dilution for CSE immunostaining in wild type and CSE knockout pancreas. Immunostaining was performed with a Rabbit specific HRP/DAB (ABC) Detection IHC Kit by Abcam, Cambridge UK as described in section 3.3.6 using serial pancreatic sections of wild type (WT) and CSE knockout (CSE KO) mice. There was some background staining in the CSE KO pancreas. The 1:1,000 dilution factor yielded the highest signal to noise ratio and was used for subsequent experiments.

8.4 Optimisation of the biotin switch protein s-sulfhydration detection assay

Figure 8.6 Varying protein and MMTS thiol blocker concentration. The purpose of this experiment was to determine if there was adequate thiol blocking by methyl methanethiosulfonate (MMTS). The experiment was carried out as described in section 2.3.8 with lysates of wild type and CSE overexpressing HEK cells. The protocol by Mustafa et al (31) reported the use of 240 μg protein with 20 mM MMTS, here I have used 160, 250 and 320 μg of protein with 20 and 100 mM MMTS. The left panel is a biotin blot that should tag s-sulfhydrated proteins as well as endogenously biotinylated proteins while the right panel is a coomasie stain of the same blot to ascertain equal protein loading between wells. Varying protein and MMTS concentration did not yield any substantial difference in staining intensity. Additionally, the CSE over expressing cells did not show any increased levels of protein s-sulfhydration.
Figure 8.7 Comparing different thiol blocker and detergent concentration. The purpose of these experiments was to determine the efficacy of thiol blocking of MMTS by using varying concentrations and comparing with another thiol blocker, NEM as well as to investigate the effect of detergent concentration. These experiments were carried out as described in section 2.3.8 with liver lysates of wild type C57BL6 mice. Both panels show biotin blots that should tag s-sulfhydrated proteins as well as endogenously biotinylated proteins. The left panel shows saturating signals in lanes without NEM or MMTS blocking due to indiscriminate labelling of thiolated proteins. Both NEM and MMTS significantly reduced this signal but there was no substantial difference between the type or concentration of blocker used. The protocol by Mustafa et al (31) reported a critical concentration of 2.5% SDS to be maintained during the experiment to ensure effective unfolding of proteins to expose protein thiols. The right panel shows no substantial change in signal with varying detergent concentrations (the MMTS + 2.5% SDS sample was lost due to accidental aspiration during a washing step). Taken together, these results suggest that maximal blocking of endogenous thiols have been achieved within the constraints of this protocol.
8.5 NFκB immunostaining

Figure 8.8 NFκB immunostaining of pancreatic sections. Immunostaining was performed on wild type mice pancreatic sections with a Rabbit specific HRP/DAB (ABC) Detection IHC Kit (Abcam, Cambridge UK) and a rabbit anti-NFκB p65 primary antibody (Abcam, Cambridge UK) diluted 1:500 as described in section 2.3.8. The left panel shows immunostaining with a hematoxylin counterstain and without the primary antibody, there was no detectable non-specific staining. The right panel shows immunostaining without hematoxylin counterstain, the positive staining was predominantly present in the cytoplasm as evidenced by the lack of nuclei staining (arrows). This is characteristic of NFκB p65 subunit which generally resides in the cytoplasm and translocates into the nucleus only upon activation.