EUKARYOTIC AND PROKARYOTIC SOURCES OF COLONIC HYDROGEN SULFIDE SYNTHESIS

MCMASTER UNIVERSITY

EUKARYOTIC AND PROKARYOTIC SOURCES OF COLONIC HYDROGEN SULFIDE SYNTHESIS

By

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ABSTRACT

Hydrogen sulfide (H_2S) is an important modulator of many aspects of digestive function, both in health and disease. Colonic tissue H_2S synthesis increases markedly during injury and inflammation and appears to contribute to resolution. Some of the bacteria residing in the colon can also produce H_2S. The extent to which bacterial H_2S synthesis contributes to what is measured as colonic H_2S synthesis is not clear. Using conventional and germ-free mice, we have delineated the eukaryotic vs. prokaryotic contributions to colonic H_2S synthesis, both in healthy and colitic mice. Colonic tissue H_2S production is entirely dependent on the presence of the cofactor pyridoxal-5'-phosphate (vitamin B_6), while bacterial H_2S synthesis appears to occur independent of this cofactor. As expected, approximately one-half of the H_2S produced by feces is derived from eukaryotic cells. While colonic H_2S synthesis is markedly increased when the tissue is inflamed, and, in proportion to the extent of inflammation, fecal H_2S synthesis does not change. Rats fed a B vitamin-deficient diet for 6 weeks exhibited significantly diminished colonic H_2S synthesis, but fecal H_2S synthesis was not different from that of rats on the control diet. Our results demonstrate that H_2S production by colonic bacteria does not contribute significantly to what is measured as colonic tissue H_2S production, using the acetate trapping assay system employed in this study.

In another study focusing on eukaryotic sources of H_2S synthesis, the contributions of three enzymatic pathways to colonic H_2S synthesis were determined in tissues taken from healthy rats and rats with colitis. The ability of colonic tissue to

inactivate H_2S was also determined. The majority of increased H_2S synthesis, in both healthy and inflamed tissue, was derived via a pyroxidal-5'-phosphate-independent pathway. Ulcerated mucosal tissue accounted for the greatest levels of H_2S synthesis, and the extent of granulocyte infiltration into the tissue did not appear to be a significant determinant of the levels of H_2S production. Inactivation of H_2S by colonic tissue occurred rapidly, but was significantly reduced in tissue from rats with colitis. Damage to colonic tissue, rather than the associated granulocyte infiltration, appears to be the major stimulus for enhanced H_2S synthesis, and multiple enzymatic pathways contribute to this synthesis. In addition to elevated production of H_2S, the ulcerated colonic tissue exhibits an impaired ability to inactivate H_2S. Together, the increased production and decreased inactivation of H_2S may contribute to promoting resolution of inflammation and repair of damaged colonic tissue.

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LIST OF ABBREVIATIONS

3MP	3-mercaptopyruvate
3MST	3-mercaptopyruvate sulfurtransferase
ASF	Altered Schaedler flora
ATP	Adenosine triphosphate
BCA	β-cyanoalanine
CAT	Cysteine aminotransferase
CBS	Cystathionine-β-synthase
CHH	O-carboxymethyl-hydroxylamine hemihydrochloride
CO	Carbon monoxide
COX	Cyclooxygenase
CSE	Cystathionine-γ-lyase
DNBS	Dinitrobenzene sulfonic acid
GF	Germ-free
H_2S	Hydrogen sulfide
IFN-γ	Interferon-gamma
IL	Interleukin
iNOS	Inducible nitric oxide synthase
MPO	Myeloperoxidase
Na_2S	Sodium sulfide
NaHS	Sodium hydrosulfide

NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
NSAID	Non-steroidal anti-inflammatory drug
P5P	Pyridoxal-5'-phosphate
PAG	DL-propargylglycine
PDE	Phosphodiesterase
SQR	Sulfide quinone reductase
TNBS	Trinitrobenzene sulfonic acid
TNF-α	Tumor Necrosis Factor-alpha
WT	Wild type

DECLARATION OF ACADEMIC ACHIEVEMENT

Experiments were conceived and designed by Kyle L. Flannigan and John L. Wallace. Kyle L. Flannigan performed all experiments. Data analysis was performed by Kyle L. Flannigan and John L. Wallace. This thesis was written by Kyle L. Flannigan with contributions from John L. Wallace.

1.0 GENERAL INTRODUCTION

1.1 The emergence of hydrogen sulfide (H_2S) in host function

Hydrogen sulfide (H_2S) is an intensely studied molecule that holds tremendous promise for new therapeutic and clinical applications (Fiorucci et al., 2007; Srilatha et al., 2006; Szabó, 2007; Wallace, 2007; Wallace and Vong, 2008). However, this was not always the case as for centuries H_2S was recognized for its characteristic smell of rotten eggs and toxic effects (Freireich, 1946; Wang, 2002). Indeed, at high concentrations (> 20 μM) H_2S can inhibit cytochrome c oxidase, impairing cellular respiration, thus acting as a metabolic toxin (Cooper et al., 2008). The toxicity of H_2S at high levels of exposure is theorized to have played a role in shaping our earlier world by causing several extinctions, including the Permian-Triassic extinction >250 million years ago (Grice et al., 2005; Wang, 2010). Even more recently, occupational exposure to high levels of H_2S has been implicated in a number of workplace deaths (Policastro and Otten, 2007**;** Reiffenstein et al., 1992).

Although often cast in a pejorative light, the current legacy of H_2S looks much more promising when compared to a century ago. In fact, the benefits of sulfide have been recognized for years as indicated by the beneficial effects of bathing in sulfur springs (Sukenik et al., 1999) and the cardioprotective effects allotted by polysulfides found in garlic (Benavides et al., 2007). Even though these benefits of exogenous sources of H_2S appeared to be of some benefit, it was against conventional thought that the

body could produce this seemingly toxic gas. This notion changed when a remarkable paper emerged in 1996 by Abe and Kimura showing that the brain produces relatively high levels of H_2S. Taking their work even further, Abe and Kimura (1996) showed that H_2S played a role in normal brain function. Using inhibitors of endogenous H_2S production and exogenously applied H_2S-releasing salts they found that H_2S facilitated the induction of hippocampal long-term potentiation thus acting as a signaling molecule in the brain (Abe and Kimura, 1996). Abe and Kimura concluded that endogenously produced H_2S was an important neuromodulator in the central nervous system (Abe and Kimura, 1996).

The newfound role for this "toxic" gas was reminiscent of The Nobel Prize-winning discovery of nitric oxide (NO) as a signaling molecule in the cardiovascular system (Ignarro et al., 1987). The discovery of NO, also once recognized as a toxic molecule, was groundbreaking in that it underscored the importance of gaseous signaling molecules in numerous physiological processes. In fact, the discovery of NO led to the designation of a new class of signaling molecules called "gasotransmitters". To be considered a gasotransmitter the gaseous molecule in question must be freely permeable to membranes, it must be enzymatically generated in the body, and its endogenous metabolism must be regulated. As well, the gas must have specific physiological functions in different systems, its effects must be mimicked by exogenous physiological concentrations, and it must trigger an intracellular signaling event or cascade (Mancardi et al., 2009**;** Wang, 2002; Wang, 2003**)**. H_2S fulfills all of these criteria

and together with NO and carbon monoxide (CO) is recognized as a gaseous mediator that contributes to host function during health and disease (Olson and Donald, 2009; Peers and Lefer, 2011; Wang, 2002).

The discovery of H_2S as a gasotransmitter has prompted an explosion of research over the past two decades. H_2S is now known to be produced throughout the body and to regulate important functions in most organs and tissues, both in health and disease. H_2S contributes to fundamental processes such as vasodilation (Zhao et al., 2004), leukocyte-endothelial adhesion (Zanardo et al., 2006), smooth muscle relaxation (Hosoki et al., 1997), and nociception (Distrutti et al., 2006; Ekundi-Valentim et al., 2010). In the digestive tract, H_2S regulates blood flow and smooth muscle tone (Fiorucci et al., 2006), modulates epithelial secretion (Schicho et al., 2006; Pouokam and Diener, 2011) and, as will be discussed in more detail below, promotes healing of ulcers and has potent anti-inflammatory actions (Wallace et al., 2007b; Wallace et al., 2009; Wallace, 2010; Wallace et al., 2010). Many fundamental questions remain regarding the regulation of the production of H_2S, its mechanisms of action, and its catabolism (Kabil and Banerjee, 2010). Answers to these questions will give further insight into the importance of this mediator and may help in developing new therapeutics (Szabó, 2007).

1.2 Endogenous production of H_2S

CBS and CSE pathways for H_2S production

The ability to synthesize H_2S is believed to be an ancient metabolic capability that has been conserved across all kingdoms of life (Kabil and Banerjee, 2010; Li et al., 2011). The generation of H_2S in mammalian tissues occurs through the enzymatic degradation of the sulfur-containing amino acid L-cysteine (Figure 1.1) (Kabil and Banerjee, 2010; Kimura, 2001; Li et al., 2011; Wang, 2004). The most intensely studied enzymes responsible for H_2S production are cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE) and are often referred to as the enzymes of the trans-sulfuration pathway. It is worth noting that as part of the trans-sulfuration pathway, CBS can condense serine and homocysteine together into cystathionine, which is converted by CSE into L-cysteine, providing the substrate for H_2S-producing reactions (Kabil and Banerjee, 2010; Li et al., 2011; Paul and Snyder, 2012). The activity of the CBS and CSE enzymes is dependent upon the presence of the biologically active form of vitamin B_6, pyridoxal-5'-phosphate (P5P) (Li et al., 2011; Wang, 2012). In the absence of P5P, CBS and CSE together cannot convert homocysteine into L-cysteine, which leads to hyperhomocysteinemia (Troen et al., 2008). As well, in the absence of P5P, CBS and CSE cannot produce H_2S.

Abe and Kimura (1996) were the first to show that these P5P-dependent enzymes were responsible for production of H_2S in animal tissues with the identification of CBS as the main producer of H_2S in the brain. Zhao et al. (2001) later found that CSE

was expressed in the cardiovascular system and was responsible for producing H_2S, which had a role in vasodilation. It is now known that CBS and CSE are differentially expressed throughout the mammalian body and are involved in the production of H_2S in many organ systems (Wang, 2012). While CBS appears to be the main source of H_2S in the central nervous system, it is believed that CSE is more heavily relied upon for H_2S production in peripheral tissues (Paul and Snyder, 2012). For example, CSE has been reported as the major H_2S-producing enzyme in the vascular smooth muscle (Hosoki et al., 1997; Zhao et al., 2001). However, the exact contribution from each of these enzymes in different organ systems remains to be fully elucidated.

In the gastrointestinal tract it appears that CBS and CSE both make important contributions to H_2S production (Martin et al., 2010; Wallace et al., 2009). Specifically, in the healthy colon, an inhibitor of CBS was able to attenuate H_2S synthesis while an inhibitor of CSE was not, suggesting that CBS is the main contributor to H_2S production in the healthy colon (Martin et al., 2010; Wallace et al., 2009). However, administration of either a CBS or a CSE inhibitor to healthy rats resulted in significant colonic inflammation (Wallace et al., 2009). Moreover, when colitis was induced in rats (through administration of a hapten), the inflamed colon displayed an increased capacity to produce H_2S, with CBS and to a lesser extent CSE being responsible for this increased production (Wallace et al., 2009).

CAT-3MST pathway for H_2S production

In 2009, Shibuya et al. reported that brain homogenates from mice deficient for the CBS enzyme were still capable of producing H_2S at levels similar to wild type mice. This production of H_2S occurred in the absence of P5P, suggesting the presence of another P5P-independent, H_2S-producing enzyme. Shibuya et al. (2009a) went on to characterize a novel pathway for H_2S production in the brain occurring through two enzymes called cysteine aminotransferase (CAT) and 3-mercaptopyruvate sulfurtransferase (3MST) (Figure 1.1). Through this pathway, CAT in the presence of α-ketoglutarate converts L-cysteine into 3-mercaptopyruvate (3MP). 3MST then converts 3MP to pyruvate and H_2S (Shibuya et al., 2009a; Shibuya et al., 2009b).

CAT and 3MST were shown to be present in neurons of the central nervous system (Shibuya et al., 2009a) and a subsequent study from the same group showed the presence of CAT and 3MST in the vascular endothelium of the thoracic aorta (Shibuya et al., 2009b). Noteworthy is the fact that CAT was found in both the cytosol and mitochondria. CAT is now classified in two forms: cCAT (cytosolic CAT) and mCAT (mitochondrial CAT) (Shibuya et al., 2009a; Shibuya et al., 2009b; Wang 2012). It is tantalizing to propose a functional relationship between these pathways, for example interplay between CAT-3MST in the endothelium vs. CSE in the smooth muscle (Shibuya et al., 2009b). However, studies examining the contributions of the CAT-3MST pathway for H_2S production relative to the CBS and CSE pathways are lacking.

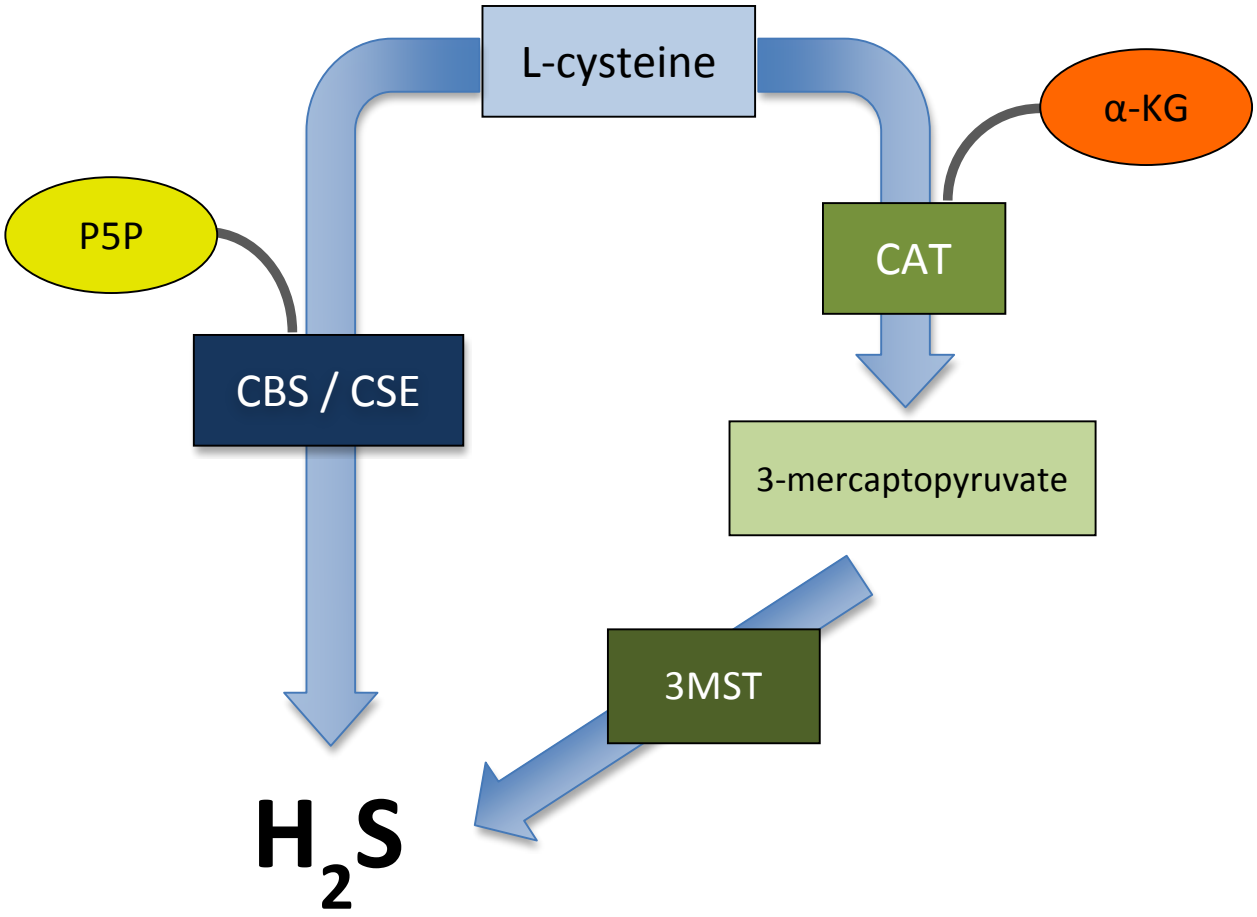

Figure 1.1 – The enzymatic pathways for hydrogen sulfide synthesis currently identified in mammalian tissues. H_2S production via cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE), is dependent on the cofactor pyridoxal-5'-phosphate (P5P). The recently identified 3rd pathway for H_2S synthesis involves the enzyme cysteine aminotransferase (CAT). In the presence of α-ketoglutarate, it can convert L-cysteine into 3-mercaptopyruvate (3MP). 3MP can then be converted into H_2S via 3-mercaptopyruvate sulfurtransferase (3MST) (adapted from Kimura, 2011).

1.3 Important tools for the study of H_2S

Along with the explosion of studies focusing on the role of H_2S in health and disease came the emergence of several new methods to study this gaseous mediator. Inhibitors of H_2S-producing enzymes are commonly employed to study endogenous production of H_2S and the enzymatic sources in different tissues. The most widely used inhibitors are O-carboxymethyl-hydroxylamine hemi-hydrochloride (CHH), β-cyanoalanine (BCA), and propargylglycine (PAG). CHH is commonly used as an inhibitor of CBS (Fiorucci et al., 2005). PAG and BCA are employed as inhibitors of CSE, although their mechanisms of inhibition differ, the former being an irreversible inhibitor and the latter a reversible inhibitor (Abeles and Walsh, 1973; Pfeffer and Ressler, 1967). These inhibitors are often used to study the pathways responsible for H_2S production in certain tissues. However, these inhibitors lack a high degree of selectivity and any observed outcomes may be unrelated to effects on H_2S synthesis.

Mice with targeted deletions of the CBS and CSE enzymes have also been developed to study enzymatic sources and physiological effects of H_2S in different organ systems. The phenotype of CSE knockout mice has only been reported in terms of the cardiovascular system (Yang et al., 2008). CBS knockout mice have been more extensively characterized, especially in terms of their liver and kidney phenotypes (Eberhardt et al., 2000; Watanabe et al., 1995). However, complete phenotypes of these mice have yet to be determined, and there is no published information on impact of the gene deletions on the gastrointestinal tract.

To examine the effects H_2S exerts of certain tissues, exogenously applied donors of H_2S have proven valuable (Li et al., 2011; Whiteman et al., 2011; Wallace, 2007; Wang 2012). Sulfide salts (e.g., NaHS and Na_2S) in aqueous solution are commonly used as H_2S-donors. One of the drawbacks with sulfide salts is their rapid release of H_2S, which in most cases is not representative of the regulated release of H_2S synthesis via the above-mentioned enzymatic pathways (Whiteman et al., 2011). Lawesson's reagent is another commonly used H_2S-donating molecule (Wallace, 2007; Wallace et al., 2007b). Newer, slow-releasing H_2S-donors, such as GYY4137 (morpholing-4-ium 4 methoxyphenyl phosphinodithiote), are used to more closely reflect enzymatic release of H_2S (Li et al., 2008; Li et al., 2009; Whiteman et al., 2010; Whiteman et al., 2011). Several H_2S-releasing derivatives of drugs, such as non-steroidal anti-inflammatory drugs (NSAIDs) and mesalamine, have also been developed (Fiorucci et al., 2007; Wallace, 2007; Wallace et al., 2007a; Wallace et al., 2010). Many of these compounds had improved efficacy and safety profiles when compared to their parent drugs and newer applications for these drugs are being examined (Fiorucci et al., 2007; Wallace, 2007; Wallace et al., 2007a; Wallace et al., 2010; Whiteman et al., 2011).

Finally, several techniques have been used to measure levels of H_2S produced by biological specimens. One of the most commonly used methods is a zinc trapping-methylene blue formation assay (Hughes et al., 2009; Stipanuk and Beck, 1982). This technique is very reproducible and allows for comparisons of the production of H_2S from large numbers of samples, in a variety of situations (e.g., health vs. disease). H_2S-

sensitive electrodes have been employed, but a lack of reproducibility limits their utility. Several techniques with higher sensitivity based upon high performance liquid chromatography (HPLC) have also been developed for H_2S measurements (Shen et al., 2011), although the equipment required and technical demand of these techniques often limits their widespread use. As well, all of the techniques mentioned to this point are used solely for *in vitro* measurements. There have been several recent articles describing novel fluorescent probes that can bind and fluorescently detect H_2S. These probes have the potential for use *in vivo* (Dufton et al., 2012; Lippert et al., 2012; Peng et al., 2011; Sasakura et al., 2011).

1.4 H_2S as a modulator of inflammation

It is now clear that H_2S is a very potent mediator of inflammation, however this area still remains a point of contention, as H_2S has been shown to be both pro- and anti-inflammatory. These discrepancies are, at least in part, due to differences in concentrations of H_2S used, different experimental models (animals vs. cell lines), and the route of administration (Rivers et al., 2012; Whiteman et al., 2010). Substantial data support that physiologically relevant concentrations of H_2S display anti-inflammatory effects. In fact, a novel H_2S-donating molecule, GYY4137 (morpholing-4-ium 4 methoxyphenyl phosphinodithiote), which slowly releases H_2S, more accurately capturing endogenous enzymatic production, had potent anti-inflammatory activities as measured by its ability to inhibit the secretion of many pro-inflammatory mediators

including TNF-α and IL1-β (Li et al., 2008; Whiteman et al., 2010). Figure 1.2 illustrates some of the key effects H_2S has with respect to modulating inflammation and injury.

One of the initial effects discovered that drove support for H_2S as an anti-inflammatory mediator was its ability to act as an endogenous modulator of leukocyte-mediated inflammation (Zanardo et al., 2006). Using sulfide salts, Zanardo et al. (2006) demonstrated that H_2S inhibited leukocyte adherence to the vascular endothelium, and the subsequent extravasation of leukocytes. These effects appeared to be a result of down-regulation of cell adhesion molecules in the vascular endothelium (Zanardo et al., 2006). Additionally, altered leukocyte-endothelial interactions have been reported in mice deficient for the CBS enzyme (Kamath et al., 2010). Mice deficient for CBS do not survive longer than 5 weeks. However, mice heterozygous for CBS displayed increased vascular permeability, reduced leukocyte-rolling velocity and increased adherence of leukocytes to the endothelium (Kamath et al., 2010).

The ability of H_2S to inhibit leukocyte adherence and extravasation is further supported by data from animal models. For example, in rat models of acute and chronic paw swelling, H_2S-releasing NSAIDs had increased edema-reducing effects when compared to parent NSAIDs (Wallace et al., 2007a; Wallace et al., 2010). Conversely, inhibitors of H_2S production triggered increased edema formation in the model of acute paw swelling (Wallace et al., 2007a). In a model of endotoxin-induced lung and liver inflammation in rats, a H_2S-releasing NSAID attenuated leukocyte extravasation and exudate accumulation (Li et al., 2007).

A large body of evidence also supports the ability of H_2S to modulate the expression of a number of inflammatory mediators. Studies performed both *in vivo* and *in vitro* by Whiteman et al. (2010) showed that H_2S-releasing molecules could inhibit endotoxin-induced secretion of the pro-inflammatory mediators IL-1β, IL-6, TNF-α, NO, and prostaglandin E_2 (PGE_2). This observed effect was attributable to the inhibition of NF-κB activation (Li et al., 2009; Whiteman et al., 2010). Similar effects have been displayed in many cell types including cultured microglia (Hu et al., 2007) and several phenotypes of rat and mouse macrophages (Dufton et al., 2012). Further *in vivo* evidence from animal models of colitis also supports the role of H_2S in mediating pro-inflammatory mediators (Fiorucci et al., 2007; Wallace et al., 2009). A H_2S-releasing mesalamine derivative suppressed the expression of mRNA for inducible nitric oxide synthase (iNOS) and several other pro-inflammatory cytokines including TNF-α, IFN-γ, and IL-1 during TNBS-induced colitis in mice, while mesalamine alone did not (Fiorucci et al., 2007). In a rat model of TNBS-induced colitis, intrarectal administration of H_2S-donors reduced disease severity, which was accompanied by a reduction in TNF-α expression and secretion (Wallace et al., 2009). Finally, studies with human tissues also reaffirm this modulatory role of H_2S as diallyl trisulfide, an H_2S-releasing compound found in garlic, decreased TNF-α expression in tissue biopsies from patients with ulcerative colitis (Bai et al., 2005).

Additional anti-inflammatory effects of H_2S include its ability to "scavenge" pro-inflammatory oxidants such as peroxynitrite ($ONOO^-$), hypochlorous acid (HOCl)

(Whiteman et al., 2004a; Whiteman et al., 2004b) superoxide, and hydrogen peroxide (Muzzaffar et al., 2008). These reactive species can promote tissue damage and the 'scavenging' effects of H_2S would be expected to alleviate inflammation. In certain situations, H_2S also imposes anti-inflammatory effects by inhibiting phosphodiesterase activity and the subsequent elevation of intracellular cyclic AMP and cyclic GMP (Bucci et al., 2010). H_2S also inhibited p38 MAP kinase, which can blunt the response to inflammatory stimuli (Hu et al., 2007; Rinaldi et al., 2006). H_2S has also been reported to stimulate the phosphorylation of protein kinase B (AKT), through interacting with phosphatidylinositol 3-kinases (PI3K) leading to anti-inflammatory signaling (Tamizhselvi et al., 2009).

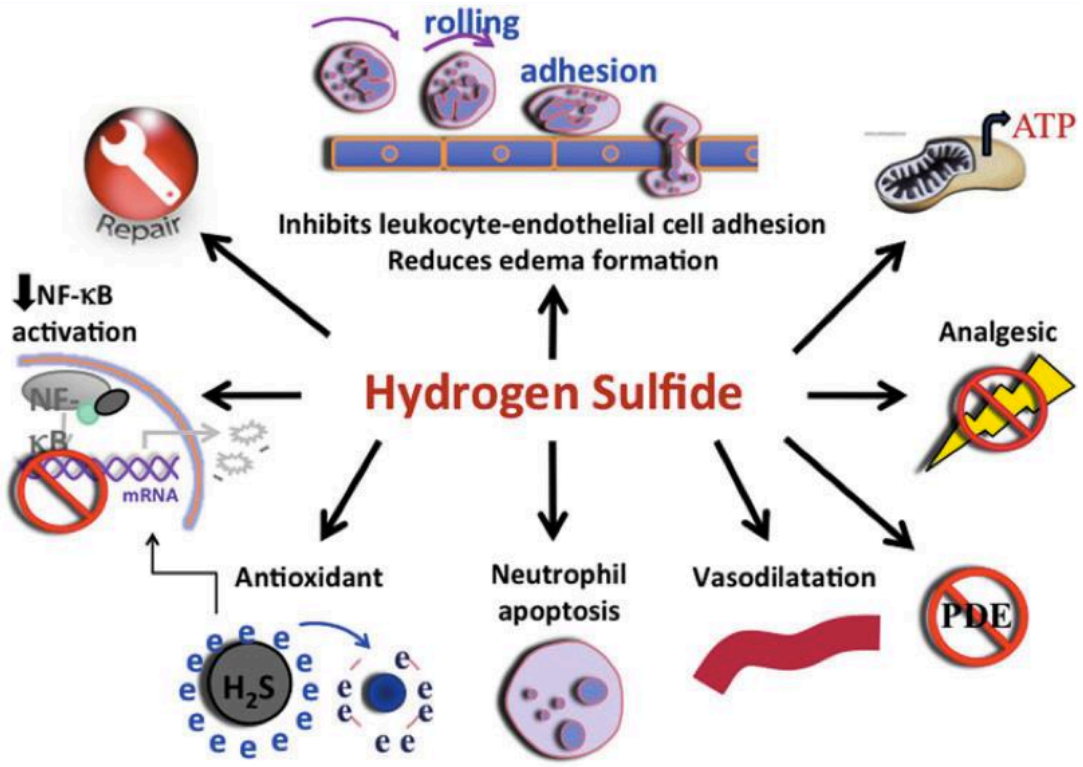

Figure 1.2 – Anti-inflammatory effects of H_2S. This figure summarizes the myriad of effects that H_2S can exert on several aspects of the inflammatory response (Wallace, 2012). H_2S is a potent inhibitor of leukocyte adherence to the vascular endothelium, preventing edema formation. H_2S has the ability to inhibit NF-κB activation and subsequent expression and secretion of pro-inflammatory mediators. H_2S may also have additional anti-inflammatory effects through the inhibition of phosphodiesterase (PDE) and its ability to act as an antioxidant. H_2S also aids in the resolution process through stimulating neutrophil apoptosis. H_2S promotes vasodilation and repair of tissue, including the gastrointestinal mucosa. Finally, H_2S can drive the production of adenosine triphosphate (ATP) in the epithelium helping to further protect this layer during times of compromise.

1.5 H_2S as a mediator of gastrointestinal mucosal defense

The mucosa of the gastrointestinal tract is frequently exposed to a wide range of insults, including acid, bile, digestive enzymes, bacteria and drugs such a non-steroidal anti-inflammatory drugs (NSAIDs) (Wallace and Ma, 2001; Wallace, 2008). The ability of the gastrointestinal mucosa to resist injury and damage produced by these agents is dependent upon a variety of factors that contribute to "mucosal defense". Several soluble mediators, such as prostaglandins and NO, are now recognized as important modulators of mucosal defense (Wallace, 2008).

Like prostaglandins and NO, H_2S has the ability to relax vascular smooth muscle, resulting in vasodilation (Zhao et al., 2001; Zhong et al., 2003). In the gastrointestinal tract, the maintenance of mucosal blood flow is important for limiting injury and promoting tissue repair after certain insults (Martin and Wallace, 2006; Wallace and Ma, 2001). Thus, the hypothesis emerged that H_2S could contribute to mucosal defense and the resistance to mucosal injury. Indeed, the ability of H_2S to function as a mediator of mucosal defense was first demonstrated by Fiorucci et al. (2005) who demonstrated the importance of H_2S for maintaining the integrity of the gastric mucosa. Several other effects of H_2S have also been shown to increase the resistance of the gastrointestinal mucosa to injury. For example, H_2S can stimulate the secretion of bicarbonate (Ise et al., 2011), which helps protect the small intestine from damage induced by the acid that enters from the stomach. H_2S can also dampen pro-inflammatory cytokine expression

and release by inhibiting NF-κB signaling, helping to reduce further damage after initial insults (Fiorucci et al., 2007; Li et al., 2007).

H_2S can also contribute to the resistance of injury by influencing the production of other mediators of mucosal defense. Cyclooxygenase-2 (COX-2) and the prostaglandins it can produce, such as PGE_2, play important roles in the maintenance of mucosal defense and in modulating mucosal inflammation (Wallace, 2008). H_2S-donors can increase prostaglandin synthesis by up-regulating expression of COX-2 (Wallace et al., 2009). On the other hand, suppression of H_2S synthesis results in leukocyte adherence and granulocyte infiltration into the mucosa (Wallace et al., 2009). This mucosal inflammation may in part be explained by the fact that H_2S inhibition led to the down-regulation of COX-2 expression, and reduced PGE_2 synthesis (Fiorucci et al., 2005; Wallace et al., 2009). These effects render the mucosa more susceptible to injury, as was demonstrated *in vivo* using a model of NSAID-induced damage in rats. Inhibition of endogenous H_2S synthesis increased the susceptibility of the mucosa to damage induced by NSAIDs (Fiorucci et al., 2005). Furthermore, exogenous H_2S-donors increased the resistance of the mucosa to injury observed following NSAID administration (Fiorucci et al., 2005). These effects appeared to be in part due to the ability of H_2S to maintain mucosal blood flow, reduce TNF-α expression and inhibit leukocyte adherence within mesenteric venules (Fiorucci et al., 2006; Wallace et al., 2009; Wallace et al., 2012; Zanardo et al., 2006).

H_2S also appears to afford protection to the gastric and intestinal mucosa during times of compromised integrity. Blackler et al. (2012) showed that H_2S-releasing NSAIDs could protect the stomach and small intestine from damage in several models of compromised mucosal defense including aged, obese, arthritic, and hypertensive rats. Mard et al. (2012) showed that administration of H_2S to rats prior to the induction of gastric ischemia-reperfusion injury was able to protect the stomach from damage. The protection afforded by H_2S may be attributable to the some of the effects mentioned above (eg., increasing mucosal blood-flow). However, there is also emerging evidence that H_2S can preserve mitochondrial function through the maintenance of oxidative phosphorylation in the face of challenges such as ischemia, thus limiting tissue injury (Elrod et al., 2007). The gastrointestinal mucosa also demonstrated the ability to use H_2S to produce ATP to conserve mitochondrial function (Goubern et al., 2007; Lagoutte et al., 2010; Wallace et al., 2012), however the ability of this effect to contribute to mucosal defense remains to be explored.

1.6 H_2S in the resolution of inflammation and injury

Resolution of inflammation and injury is an active process coordinated by a variety of soluble mediators. Lipoxins, certain prostaglandins, annexin-A1, and IL-10 are among the mediators that have been shown to promote resolution of inflammation (Serhan et al., 2007). H_2S is now recognized as another mediator that promotes the

resolution of inflammation and injury (Wallace et al., 2007b; Wallace et al., 2009; Wallace, 2010; Wallace et al., 2012).

H_2S prevents leukocyte adherence to the vascular endothelium, as well as leukocyte extravasation (Zanardo et al., 2006). It also induces neutrophil apoptosis (Mariggio et al., 1998), an important early event in the resolution process (Serhan et al., 2007). H_2S can further promote resolution of inflammation through effects on macrophages (Dufton et al., 2012). During resolution, infiltrating macrophages undergo a switch from a pro-inflammatory to an anti-inflammatory phenotype, promoting processes such as those required for clearing noxious agents and apoptotic neutrophils (Serhan et al., 2007; Wallace, 2012). In cultured macrophages, H_2S significantly increased the rates of phagocytosis and chemotaxis, while inhibiting endotoxin-induced TNF-α production (Dufton et al., 2012). Also consistent with a shift in macrophage phenotype was the observed ability of H_2S to significantly reduce granulocyte infiltration while maintaining macrophage numbers in a model of mouse peritonitis (Dufton et al., 2012). H_2S can further affect the resolution process by influencing the production of a number of other mediators of resolution. As mentioned above, H_2S can up-regulate the expression of COX-2 and the production of certain prostaglandins (Serhan et al., 2007; Wallace et al., 2012). As well, a slow releasing H_2S-donor was able to up-regulate release of the anti-inflammatory cytokine IL-10 from cultured macrophages (Li et al., 2009). Finally, Brancaleone et al. (2011) have demonstrated that H_2S can activate the annexin-A1 pathway, an anti-inflammatory pathway important for resolution and mucosal

integrity. H_2S caused the mobilization of annexin-A1 from the cytosol to the cell membrane of neutrophils, a hallmark sign of annexin-A1 pathway activation. Furthermore, H_2S down-regulated the expression of inflammatory pathways (iNOS and COX-2) in lipopolysaccharide-stimulated macrophages from wild-type mice but not in macrophages obtained from annexin-A1-deficient mice (Brancaleone et al., 2011).

H_2S has also been shown to promote healing and resolution *in vivo*. For example, Wallace et al. (2007) demonstrated that the ability of gastric tissue to produce H_2S markedly increased after the induction of gastric ulceration using acetic acid. Increased levels of H_2S production coincided with increased expression of both CBS and CSE enzymes. Inhibitors of H_2S synthesis significantly impaired ulcer healing, while administration of H_2S-donors (Lawesson's reagent or an H_2S-releasing NSAID derivative) promoted the healing of gastric ulcers. L-cysteine, the precursor for H_2S production, also significantly enhanced ulcer healing. Part of these effects may have been a result of increased mucosal blood-flow at the margins of ulcers (Wallace et al., 2007b). Additional effects that may have contributed to the resolution of injury include the ability of H_2S to promote angiogenesis, thus further increasing blood flow to sites of injury (Coletta et al., 2012; Szabó and Papapetropoulos, 2011; Wallace, 2007). As well, H_2S can act as an energy source for mitochondria, allowing for maintenance of ATP production during periods of hypoxia, as may occur in ulcerated tissue (Elrod et el., 2007; Wallace et al., 2012).

Using a model of colitis, Wallace et al. (2009) were the first to describe H_2S as an important modulator in the resolution of colonic inflammation and ulceration. As in the model of gastric ulceration, there was an increased capacity of the rat colon to produce H_2S during a bout of colitis. This increase occurred as early as 6 hours after induction of colitis and was maximal at day 3. After 28 days, when colitis was largely resolved, levels of H_2S production had returned to control levels. Inhibition of H_2S production resulted in detrimental effects in healthy rats and in rats with colitis. In healthy rats, inhibition of H_2S lead to significant thickening of the submucosa as well as increased granulocyte infiltration into the tissue as measured by MPO activity. Inhibition of H_2S synthesis in rats with colitis resulted in significant mortality, mostly a result of bowel wall perforation. Furthermore, H_2S-donors (NaHS and Lawesson's reagent) administered intrarectally during a bout of colitis significantly decreased the severity of disease when compared to vehicle-treated rats (Wallace et al., 2009). These *in vivo* data support a role of H_2S in the resolution of inflammation/ injury and highlight the potential clinical value of H_2S in gastrointestinal ulceration and inflammatory bowel disease (IBD).

1.7 The colonic microbiota as a source of H_2S

Over the last decade, technological advances in gene sequencing have driven a substantive increase in research on the role of the gut microbiota in health and disease. It is becoming clear that this complex bacterial community that colonizes the gastrointestinal tract during and shortly after birth should be considered when

examining gut physiology (Eckburg et al., 2005; Neish, 2009). Members of this gut microbiota are capable of producing many molecules and metabolic by-products in the lumen of the gastrointestinal tract that can influence many different aspects of gut function (Neish, 2009; Sartor, 2008). Pertinent to our studies is the fact that certain members of the gut microbiota are capable of producing H_2S (Blachier et al., 2010; Sartor, 2008). The ability to synthesize H_2S is believed to be an ancient metabolic capability as it has been conserved across all kingdoms of life (Kabil and Banerjee, 2010). This fact makes it very easy to understand why the vast numbers of bacterial species harbored in the gut can create an environment of relatively high H_2S concentrations.

The exact role of bacterial-derived H_2S in influencing gut physiology is not fully known. It has been reported that H_2S can inhibit the oxidation of n-butyrate, the main source of energy for colonocytes, and has been implicated in the etiology of ulcerative colitis (Babidge et al., 1998). However there is a lack of compelling evidence that H_2S causes damage to the colonic epithelium. In fact, more recent reports suggest that colonocytes are well adapted for using H_2S as a metabolic fuel (Goubern et al., 2007; Lagoutte et al., 2010). The ability to use H_2S as a metabolic fuel during times of oxygen deprivation may actually benefit the epithelium, aiding in processes such as healing (Wallace et al., 2012).

Sulfur-containing amino acids, including L-cysteine can escape digestion in the small intestine and reach the colon where they can be converted to H_2S by the endogenous microbiota (Evenepoel et al., 1999; Gaudichon et al., 2002). *Escherichia coli,*

Salmonella enterica, Clostridia, and *Enterobacter aerogenes,* all of which can be found in the large intestine, are among the bacteria that can metabolize cysteine to H_2S (Blachier et al., 2010). As further work is done to characterize the human gut microbiota, the number of bacteria capable of converting cysteine into H_2S, will only grow. H_2S is also produced in the colon by sulfate-reducing bacteria that have the ability to reduce sulfate and other sulfur containing compounds to H_2S (Blachier et al., 2010). H_2S production by sulfate-reducing bacteria does not use cysteine and instead a number of dissimilatory reactions can reduce sulfate into H_2S (Nakamura et al., 2010).

The concentration of H_2S in the lumen of the human and mouse colon has been reported by some investigators to be in the millimolar range (Babidge et al., 1998; Roediger et al., 1993). However, others have provided evidence that most of the sulfide content in the colonic lumen exists in a bound form in fecal material (Levine et al., 1998, Picton et al., 2002), so levels of "free" H_2S are more likely to be in the micromolar range (Levine et al., 1998). Although uncertainty remains about the exact concentrations of H_2S in the colon, the possibility that bacteria of the colon contribute to what we measure as colonic H_2S synthesis still remains.

1.8 Enzymatic metabolism of H_2S

With the explosion of interest in H_2S and its ability to be produced in the body, came the need to understand how this molecule is regulated. This regulation includes the metabolism and inactivation of H_2S to extinguish its signaling effects in tissues. It is

clear that local tissue concentrations are not only dependent upon enzymatic production but are also influenced by the rate of H_2S metabolism (Kimura, 2012). Indeed, there is now emerging evidence that H_2S-oxidation enzymes are differentially expressed throughout the mammalian body (Kimura, 2012; Linden et al., 2011).

These enzymatic pathways for H_2S catabolism are also of interest because bacteria in the gut are major contributors to luminal concentrations of H_2S. Although luminal concentrations were originally reported at high concentrations (Babidge et al., 1998; Roediger et al., 1993) and considered detrimental to colonocytes (Babidge et al., 1998), it is now understood that levels of H_2S in the lumen are not as high as first reported (Levine et al., 1998). In fact, most of the H_2S that is produced in the lumen is bound to fecal material and therefore unavailable to diffuse through the epithelial layer (Levine et al., 1995; Picton et al., 2002). Any unbound H_2S that is "free" for diffusion will encounter the H_2S-oxidation enzymes that are highly expressed in the intestinal mucosa (Goubern et al., 2007; Linden et al., 2012; Mimoun et al., 2012). Virtually all H_2S that is free to diffuse across the apical membrane of enterocytes and colonocytes is rapidly oxidized to thiosulfate (Goubern et al., 2007; Levitt et al., 2002). This process occurs in the mitochondria mainly through the enzyme sulfide quinone reductase (SQR) (Linden et al., 2012). Not only is this enzyme capable of rapidly oxidizing and thus "detoxifying" luminal H_2S, but this process of oxidation to thiosulfate drives the production of ATP through reverse election transfer in colonocytes (Goubern et al., 2007; Lagoutte et al., 2010). This capacity is regarded as an ancestral trait adapted to low-light, low-oxygen

conditions and pre-dates photosynthesis (Theissen et al., 2003). Therefore, the intestinal mucosa can be viewed not only as a physical barrier to luminal contents, but also as a metabolic barrier. However, because this barrier is important for regulating local concentrations of H_2S, any physical breach, such as those observed during experimental colitis may alter local tissue concentrations of H_2S and influence gastrointestinal function.

1.9 Objectives

The fact that endogenous microbes of the gut, especially the colon, can produce H_2S raises the possibility that what we measure as colonic H_2S synthesis, may actually be coming from bacteria residing in the colon. This question becomes even more pertinent when considering the increased capacity of the colon to produce H_2S during inflammation. The first section of this thesis focuses on this question by addressing the prokaryotic contributions to colonic H_2S synthesis.

While examining prokaryotic sources of H_2S, we became more interested in the eukaryotic sources of H_2S synthesis. Although there is clear evidence that H_2S is involved in tissue repair throughout the gastrointestinal tract and promotes the resolution of colitis, very little is known about the enzymatic and cellular sources of this mediator. Even less is understood about the catabolism of H_2S in the gastrointestinal tract. The second section of this thesis examined enzymatic, catabolic and cellular sources of H_2S synthesis. Together, the sections of this thesis will cover the following objectives:

1. To examine the contributions of the colonic microbiota to what is measured as colonic H_2S in both the healthy and inflamed colon.

2. To investigate the presence and function of a P5P-independent pathway for H_2S synthesis, involving the enzymes CAT and 3MST, in both the healthy and inflamed colon.

3. To get a better understanding of which cells are important in the production of H_2S synthesis in the healthy and inflamed colon.

4. To explore the possibility that ulceration may affect H_2S-inactivation pathways affecting local concentrations of H_2S in the inflamed/ damaged colon.

2.0 GENERAL METHODS AND MATERIALS

Examining the production of hydrogen sulfide (H_2S) in the healthy colon and in the colon during inflammation/ injury was a crucial aspect of all following studies. Therefore, many methods and material such as the induction of colitis, the assessment of colonic inflammation and damage, and the measurement of H_2S synthesis were employed throughout. This chapter describes in detail the methods that were common to the following studies. Methods and materials pertinent to a specific study are detailed in the appropriate chapters.

2.1 Animals

All animals, both mice and rats, were housed in plastic cages and maintained under controlled temperature (20°C), humidity (60%-70%), and light cycle (12 h:12 h light-dark). Animals, unless otherwise noted, were fed standard laboratory chow and water *ad libitum*. Germ-free (GF) mice and mice with altered Schaedler flora (ASF) were maintained in McMaster's Axenic Gnotobiotic Unit to maintain germ-free and ASF conditions, respectively. All experimental protocols were approved by the Animal Research Ethics Board at McMaster University, and adhered to the guidelines established by the Canadian Council on Animal Care.

2.2 TNBS and DNBS models of experimental colitis

Hapten-induced models of colitis are widely used to examine intestinal inflammation. The trinitrobenzene sulfonic acid (TNBS) model of colitis, involves the use of ethanol to disrupt the colonic epithelial barrier, allowing TNBS to enter the lamina propria where it drives immune-mediated tissue injury and inflammation (Morris et al., 1989). In recent years, access to TNBS for laboratory use has become increasingly limited. Dinitrobenzene sulfonic acid (DNBS) has proven to be a suitable substitute for TNBS. DNBS produces colonic inflammation and ulceration indistinguishable from TNBS (Wallace et al., 1995). For the initial study in Chapter 3, TNBS was used to induce colonic inflammation in mice. However, depletion of our supply and the inability to obtain TNBS forced us to move to the DNBS model of colonic inflammation in rats for the subsequent study. Both methods for the induction of colitis are outlined here.

TNBS administration in mice

TNBS administration was performed after lightly sedating the mice with isoflurane. A 5FG pediatric feeding tube catheter was inserted via the rectum 2.5 cm into the colon. The TNBS was administered slowly (~5 sec) while applying pressure around the rectum to prevent leakage. Pressure was maintained while removing the catheter and the rectum was held closed for 4 min after TNBS administration. As outlined in the following chapter, different doses of TNBS were used to induce varying severities of disease.

DNBS administration in rats

DNBS was administered using a 5FG pediatric feeding tube catheter inserted via the rectum 8 cm into the colon. A fulminant colitis was induced by administering 0.5 mL of 60 mg/mL DNBS in 50% ethanol. After injecting DNBS, pressure was applied around the rectum of rats for 30 sec to prevent leakage. Rats were fully conscious during the induction procedure and care was taken to minimize the level of discomfort and stress.

2.3 Assessment of colonic damage and ulceration

Macroscopic colonic damage was assessed using a slightly modified version of that described previously (Wallace et al., 1990), as outlined in Table 2.1. After mice and rats were euthanized by an overdose of isoflurane, the colons was excised, opened along the mesenteric border and washed clean of fecal material. Damage was scored blindly. The criteria for colonic damage included the presence of hyperemia, bowel wall thickening, the number of sites of inflammation, and the length of ulcerated segments. Colon thickness was measured using digital calipers. In addition, adhesions, diarrhea, and rectal bleeding were scored as part of the overall macroscopic damage score. The sum of the scores from each category was taken as a total colonic damage score.

2.4 Measurement of myeloperoxidase activity

Colonic inflammation was also assessed by the measurement of myeloperoxidase (MPO) activity, as first described by Bradley et al. in 1982. MPO is an enzyme found in all cells of myeloid origin, but the greatest concentrations are found in the azurophilic granules of neutrophils. It has been used extensively as a quantitative index of granulocyte infiltration (Bradley et al., 1982; Wallace et al., 1990). Samples of the distal colon were excised from areas of ulceration or inflammation making sure to incorporate a margin of macroscopically normal tissue. All tissue was snap-frozen in liquid nitrogen immediately after collection and stored at -80°C until performing assays for MPO activity. Absorbance was measured at 450 nm over 30 second intervals (SpectraMax M3, Molecular Devices Corp., Sunnyvale, CA, USA) and converted to a K_{max} value using SoftMax Pro 5.4 software. These values were then converted to average units of MPO activity per mg of tissue.

2.5 Measurement of hydrogen sulfide production

The ability of tissue to produce H_2S was measured using a modified version (Qu et al., 2006; Wallace et al., 2007b) of an assay first described by Stipanuk and Beck (1982). This method used zinc acetate to trap H_2S which was subsequently acidified with N,N'-dimethyl-p-phenylenediamine (NNDP) and ferric (III) chloride ($FeCl_3$) to produce methylene blue. The colour intensity of the methylene blue formed was indicative of the concentration of trapped H_2S and was detected by spectrophotometry to give relative levels of H_2S. This assay was performed with various types of tissue homogenates. All

tissue was homogenized in ice-cold 50 mM potassium phosphate buffer (pH 8.0; 12% w/ v), with a Polytron homogenizer (Kinematica, Bohemia, NY, USA). Tissue homogenates (0.5 mL), buffer (0.4 mL) and substrates and/ or inhibitors of the H_2S reaction were added to large scintillation vials resulting in a final volume of 1 mL. For initial studies L-cysteine (10 mM) and P5P (2 mM) were added to the reaction mixture. Later studies used other substrates and inhibitors. A smaller 2-mL tube containing a piece of filter paper (0.5 by 1.5 cm) soaked with zinc acetate (1%; 0.3 mL) was placed inside the larger vial. The larger vials were then flushed with nitrogen gas for 20 sec and capped with an airtight serum cap. The vials were then transferred to a 37°C shaking water bath for 90 min. Next, samples were transferred to wet ice for 10 min, after which trichloroacetic acid (TCA; 50%; 0.4 mL) was injected into the reaction mixture through the serum cap. The mixture was then transferred to a 50°C shaking water bath for 60 min to allow for the trapping of evolved H_2S by the zinc acetate. The zinc acetate used to trap H_2S did not come into contact with the homogenized tissue at any point during the reaction. The serum cap was then removed and NNDP (20 mM; 50 μL) in 7.2 M HCl and $FeCl_3$ (30 mM; 50 μL) in 1.2 M HCl were added to the inner tube containing zinc acetate. Samples were left to stand in the dark for 20 min. Absorbance at 670 nm was measured with a microplate reader (Molecular Devices Corp., Sunnyvale, CA, USA). A calibration curve of absorbance versus H_2S concentration was generated using NaHS of varying concentrations.

2.6 Isolation of rat peritoneal neutrophils

To collect activated neutrophils in order to examine their capacity to produce H_2S, rats were lightly sedated with isoflurane, and 20 mL of a 1% solution of glycogen (from oyster, type II) was injected intraperitoneally (20-G needle). Four hours later the rats were euthanized by an overdose of isoflurane, and a small incision was cut in the abdomen of the rat to allow access to the peritoneum. Heparinized saline (20 mL; 25 U/ mL) was injected through the incision, and the abdomen of the animal was manipulated gently to allow the mixing of peritoneal contents. Peritoneal fluid was collected using a pediatric 5FG feeding tube. The peritoneal fluid was centrifuged (15 x g, 15 min), and the resulting pellet was re-suspended in 10 mL of heparinized saline (25 U/ mL). Further dilutions of these cells were made in isotonic saline. These various concentrations of cells were assayed for MPO activity and H_2S synthesis following the same procedures as described above.

2.7 Statistical analysis

All data are expressed as means ± SEM. Linear regression analyses were performed using GraphPad Prism version 5.0. Comparisons of data were performed using a one-way analysis of variance and the Dunnett's multiple-comparison test. In certain circumstances groups of data were compared to one another using a one-way analysis of variance and the Neuman-Keuls test. An associated probability (P value) of < 5% was considered significant.

2.8 Materials

Animals housed at McMaster University were obtained from Charles Rivers Laboratories (Saint-Constant, QC, Canada). Dinitrobenzene sulfonic acid (DNBS), L-cysteine, pyridoxal-5'-phosphate (P5P), α-ketoglutarate, L-aspartate, O-carboxymethyl-hydroxylamine hemihydrochloride (CHH), potassium cyanide, trichloroacetic acid (TCA), ferric (III) chloride, N,N'-dimethyl-p-phenylenediamine sulfate salt (NNDP), oyster glycogen: type II, zinc acetate, and NaHS were obtained from Sigma-Aldrich (Oakville, Ontario, Canada). Heparin sodium (for heparinized saline) was obtained from Leo Pharma Inc. (Thornhill, Ontario, Canada). Isoflurane was obtained from Abbott Laboratories (Montreal, Quebec, Canada).

Table 2.1 – Criteria for scoring colonic damage, ulceration and inflammation

Score	Appearance
0	Normal
1	Localized hyperemia, no ulcers
2	Ulceration without hyperemia or bowel wall thickening
3	Ulceration with inflammation at 1 site
4	Two or more sites of ulceration and inflammation
5	Ulceration at multiple sites or extending >1 cm along the length of the colon
6-10	When an area of damage extended >2 cm along the length of the colon, the score was increased by 1 for each additional cm of involvement

In addition, the score was increased by 1 or 2 if there were mild or severe adhesions, respectively, by 1 if diarrhea was evident, and by 1 if rectal bleeding was evident. The maximum colon thickness (mm) was also added to the score.

3.0 EUKARYOTIC AND PROKARYOTIC CONTRIBUTIONS TO COLONIC HYDROGEN SULFIDE SYNTHESIS

3.1 INTRODUCTION

While often regarded as a toxic gas (Freireich, 1946), hydrogen sulfide (H_2S), like nitric oxide (NO) and carbon monoxide (CO), is increasingly recognized as an endogenous gaseous mediator with many physiological functions in the body (Li et al., 2007; Wang, 2002; Fiorucci et al., 2006). H_2S may also have considerable therapeutic potential (Fiorucci et al., 2007; Srilatha et al., 2006; Szabó, 2007; Wallace, 2007). H_2S is produced in most mammalian tissues and contributes to fundamental processes such as vasodilation (Zhao et al., 2001), neuromodulation (Abe and Kimura, 1996; Kimura, 2002), leukocyte-endothelial adhesion (Zanardo et al., 2006), smooth muscle relaxation (Hosoki et al., 1997), and nociception (Distrutti et al., 2006; Ekundi-Valentim et al., 2010). In the gastrointestinal tract, H_2S has been shown to enhance ulcer healing (Wallace et al., 2007b), to promote the resolution of colitis (Wallace et al., 2009), and to contribute to gastric mucosal defense (Fiorucci et al., 2005; Fiorucci et al., 2006; Wallace, 2010).

H_2S is produced in mammalian tissue from the amino acid cysteine. Catabolism of cysteine into H_2S, pyruvate, and ammonia can occur via two pyridoxal-5'-phosphate (P5P)-dependent enzymes: cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE) (Li et al., 2010; Wang, 2002). The synthesis of H_2S throughout the gastrointestinal tract of mice and rats has been characterized using the zinc acetate-trapping method (Martin et al., 2010). This same technique was employed to measure H_2S production in the inflamed rat colon (Wallace et al., 2009). H_2S was found to be increased 100-fold

within 24 hours of induction of colitis via administration of trinitrobenzene sulfonic acid (TNBS). Inhibition of H_2S synthesis resulted in impaired resolution of the inflammation, while administration of H_2S-donors accelerated repair (Wallace et al., 2009).

Bacteria residing in the lumen of the gastrointestinal tract can also produce H_2S, and recent studies suggest that colonocytes and other cells can use it as an energy source (Goubern et al., 2007; Lagoutte et al., 2010). The concentration of H_2S in the lumen of the human and mouse colon has been reported by some investigators to be in the millimolar range (Babidge et al., 1998; Roediger et al., 1993). However, others have provided evidence that most of the sulfide content in the colonic lumen exists in a bound form in fecal material (Levine et al., 1995; Picton et al., 2002), so levels of "free" H_2S are more likely to be in the micromolar range (Levine et al., 1995). The extent to which H_2S produced by luminal bacteria can gain access to the colonic mucosa is not clear. While H_2S that is not bound to fecal material can freely diffuse across the apical membrane of a colonocyte (Mathal et al., 2009), virtually all absorbed H_2S is oxidized to thiosulfate (Goubern et al., 2007; Levitt et al., 2002). This oxidation primarily occurs in colonocytes (Goubern et al., 2007), keeping H_2S concentrations at non-toxic levels (Lagoutte et al., 2010).

One concern with measurements of colonic H_2S synthesis is the potential for contamination of tissue samples by bacteria. Thus a portion of what is measured as colonic H_2S synthesis could in fact be bacterial H_2S synthesis. The present study was undertaken to determine the extent to which H_2S produced by colonic bacteria might

contribute to what is measured as "colonic" H_2S synthesis using the widely employed zinc acetate-trapping assay (Stipanuk and Beck, 1982). To do this, we used conventional and germ-free mice and examined colonic H_2S synthesis in physiological and pathophysiological conditions. The dependence of tissue vs. fecal H_2S synthesis on the presence of P5P and L-cysteine in the assay was also examined.

3.2 METHODS AND MATERIALS

Animals

Male, Swiss Webster, NIH Swiss and NMRI mice and male, Wistar rats were housed in plastic cages and maintained under controlled temperature (20°C), humidity (60%-70%), and light cycle (12 h:12 h light-dark). The animals were fed standard laboratory chow and water *ad libitum*. Germ-free mice of the same strains were derived and maintained germ-free as previously described (Slack et al., 2009; Smith et al., 2007) at McMaster's Axenic Gnotobiotic Unit. Swiss Webster, NIH Swiss and NMRI mice with altered Schaedler flora (ASF, Taconic) (Dewrist et al., 1999) were used as colonized counterparts to germ-free mice. Mice colonized with ASF were kept in individually ventilated microisolator-cages in McMaster's Axenic Gnotobiotic Unit. All experimental protocols were approved by the Animal Research Ethics Board at McMaster University and adhered to the guidelines established by the Canadian Council on Animal Care.

Experimental Colitis

Colitis was induced in conventionally housed mice using trinitrobenzene sulfonic acid (TNBS) (Morris et al., 1989). A mild colitis was induced by intrarectal administration of 100 µl of a 10 mg/ mL solution of TNBS in 40% ethanol. A more severe colitis was induced by administering 100 µl of a 20 mg/ mL solution of TNBS in 40% ethanol. Macroscopic colonic damage was assessed using a slightly modified version of that described previously (Wallace et al., 1990), as outlined in Table 2.1. Colonic inflammation was also assessed by the measurement of myeloperoxidase (MPO) activity, as first described by Bradley et al. in 1982. MPO is an enzyme found primarily in the azurophilic granules of neutrophils, and has been used extensively as a quantitative index of granulocyte infiltration (Bradley et al., 1982; Wallace et al., 1990).

Isolation of rat peritoneal neutrophils

To examine the capacity of neutrophils to produce H_2S, activated neutrophils were collected from the rat peritoneum as previously described (Boughton-Smith et al., 1988). Peritonitis was induced using 20 mL of a 1% solution of glycogen (from oyster, type II) injected intraperitoneally (20-G needle). Peritoneal fluid was collected 4 hours after. The peritoneal fluid was centrifuged (15 x g, 15 min), and the resulting pellet was resuspended in 10 mL of heparinized saline (25 U/ml). Further dilutions of these cells were made in isotonic saline. These various concentrations of cells were assayed for MPO activity and H_2S synthesis following the same procedures as described above.

Measurement of Hydrogen Sulfide Production

The capacity for samples of colon and feces to produce H_2S was measured using a slightly modified version (Wallace et al., 2007b) of a previously described assay (Stipanuk et al., 1982). Pyridoxal-5'-phosphate (P5P; 2 mM) and L-cysteine (10 mM) were generally included in the reagent mixture. However, in some experiments, P5P, L-cysteine, or both, were excluded from the assay.

Effects of Vitamin B Deficiency

Rats were provided one of two diets for 6 weeks. One diet lacked vitamin B_6, vitamin B_{12} and folate, and has previously been shown to induce hyperhomocysteinemia (Troen et al., 2008). As the enzymes for H_2S synthesis require vitamin B_6, this diet would be expected to cause an impairment of H_2S synthesis. The control diet was identical, but contained the above-mentioned vitamins and folate. Both diets were prepared by Harlan Teklad (Madison, WI, USA) and contained 1% sulfathiozole (10 g/kg diet) to inhibit folate formation by gut bacteria (Troen et al., 2008).

3.3 RESULTS

Colonic H_2S synthesis is similar in germ-free and colonized mice

As shown in Fig. 3.1, colonic H_2S synthesis was similar in Swiss Webster mice that were germ-free and those colonized with ASF. Similar findings were obtained with two other strains of mice (NMRI and NIH), but the sample sizes in the germ-free groups were small (3 and 2, respectively). There was also no significant difference in liver H_2S synthesis between germ-free and ASF-colonized mice (1,172 ± 207 vs. 826 ± 143 nmol/ g/ h, respectively).

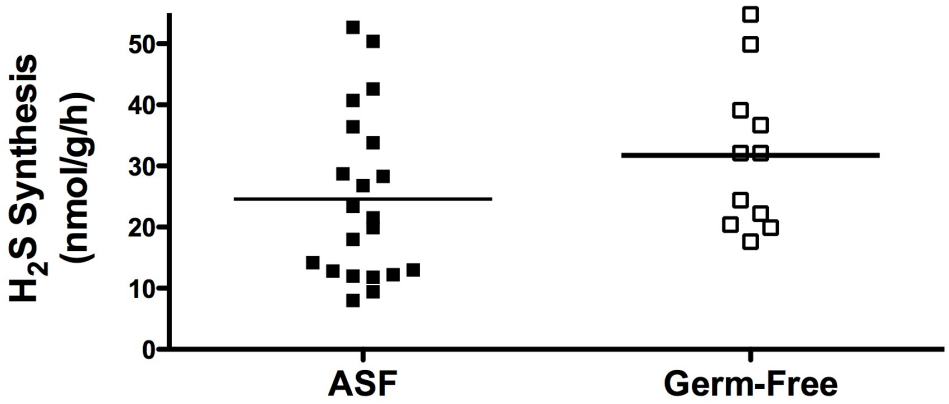

Figure 3.1 – Colonic hydrogen sulfide production in germ-free vs. ASF colonized mice. Colonic H_2S synthesis did not differ significantly between germ-free mice and the same strains of mice colonized with altered Schaedler flora (ASF). Both groups include Swiss Webster, NIH Swiss, and NMRI mice.

Eukaryotic and prokaryotic contributions to fecal H_2S synthesis

Both colonic tissue H_2S synthesis and fecal H_2S synthesis are dependent on the presence of L-cysteine in the reaction mixture (Fig. 3.2*A*). However, while colonic tissue H_2S synthesis is completely P5P-dependent, fecal H_2S production is only 50% dependent on the presence of P5P. This is likely a reflection of bacterial cells comprising only 50% of fecal mass (Tannock, 2007). Thus the P5P-independent portion of fecal H_2S synthesis likely represents the prokaryotic contribution. This conclusion is supported by the observation that fecal H_2S synthesis in germ-free mice is only ~50% of that observed in colonized mice (Fig. 3.2*B*). It is noteworthy that a portion of fecal H_2S synthesis in germ-free mice was not dependent on the presence of P5P in the reaction mixture.

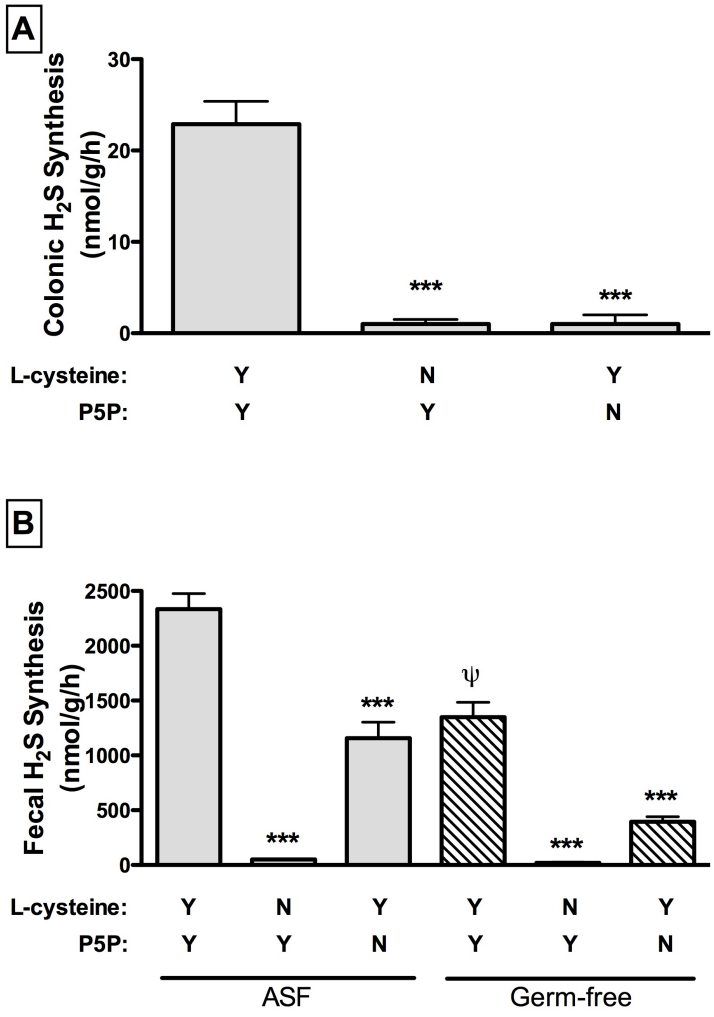

Figure 3.2 – Requirement for L-cysteine and pyridoxal-5'-phosphate (P5P) in H_2S synthesis by mouse colonic tissue and feces. The presence of L-cysteine or P5P is indicated by "Y" and the absence by "N." *Panel A*: Colonic H_2S synthesis in NIH Swiss mice. H_2S synthesis is almost completely dependent on the presence of both L-cysteine and P5P (***P < 0.001 vs. the corresponding group with both L-cysteine and P5P). *Panel B*: Fecal H_2S synthesis in NIH Swiss mice that were raised germ-free compared with NIH mice colonized with ASF. While completely dependent on the presence of L-cysteine,

fecal H_2S synthesis in ASF and germ-free mice is only ~50% and ~70% dependent on P5P, respectively (***P < 0.001 vs. the corresponding group with both L-cysteine and P5P; ψ P < 0.05 vs. the corresponding ASF group). Note: colonic H_2S synthesis did not differ significantly between conventionally housed and ASF-colonized NIH Swiss mice (data not shown). Each bar represents the mean ± SEM of 4-6 samples.

Colonic damage/inflammation affects colonic but not fecal H_2S synthesis.

Induction of colitis resulted in increases in colonic H_2S synthesis, with the greatest increases seen in animals with the most severe colitis (Fig. 3.3, *A*–*C*). Indeed, colonic H_2S synthesis correlated very well with tissue MPO activity (a marker of granulocyte infiltration) (Fig. 3.3*E*). In contrast, fecal H_2S synthesis was not altered when the colon was inflamed, compared with healthy controls, and there was no such correlation between tissue MPO activity and fecal H_2S synthesis (Fig. 3.3, *D* and *F*). Given the correlation between tissue MPO activity and tissue H_2S synthesis, it was possible that neutrophils were a significant source of the measured H_2S. To explore this, we measured H_2S synthesis by neutrophils harvested from the peritoneum (following i.p. injection of oyster glycogen). As shown in Fig. 3.4, there was no correlation between the concentration of neutrophils and the amount of H_2S produced (although the concentration of neutrophils did correlate very well with MPO activity; $R^2 = 0.98$; $P < 0.001$).

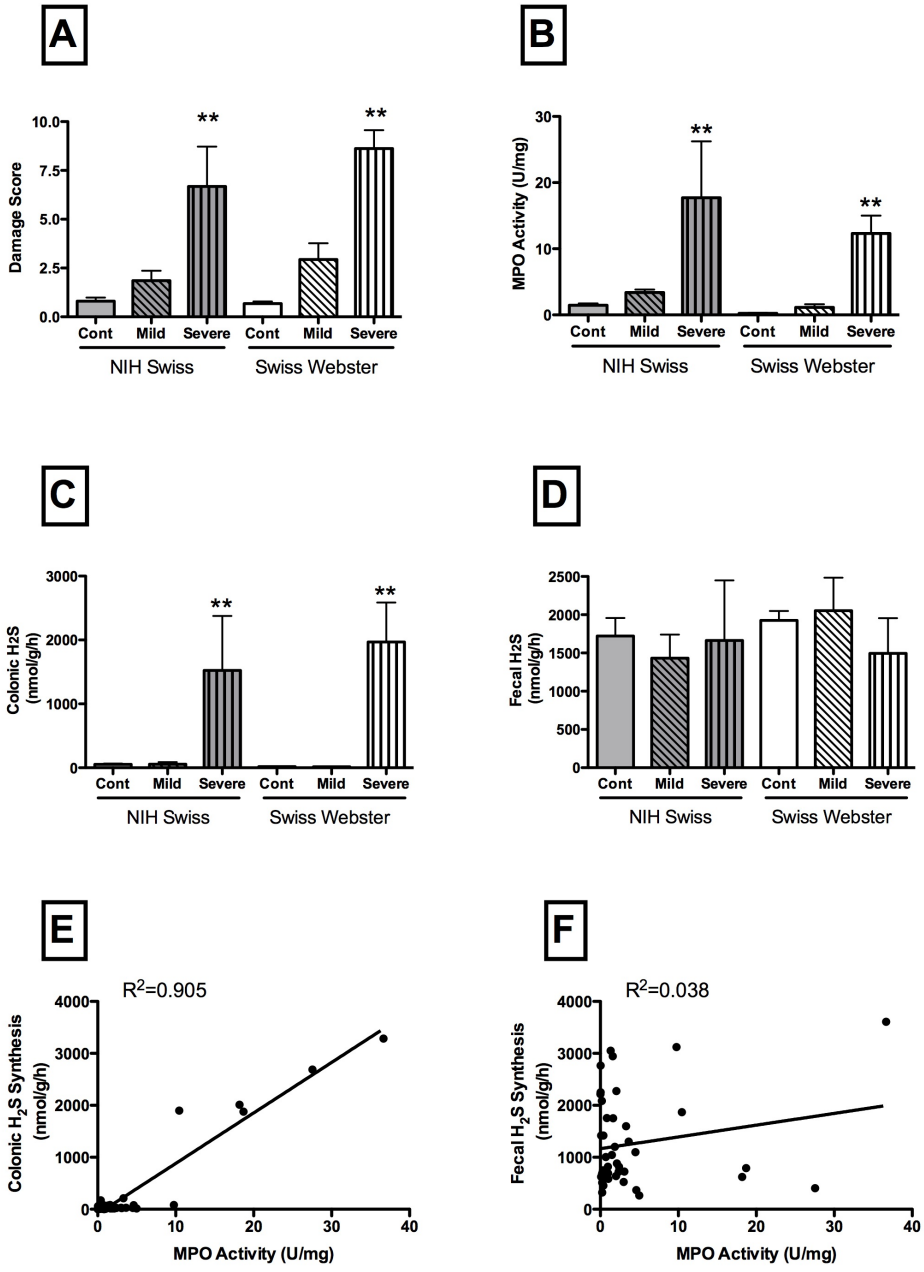

Figure 3.3 – Differences in colonic vs. fecal H_2S synthesis in healthy mice or mice with mild or severe colitis (induced by trinitrobenzene sulfonic acid). Colonic H_2S synthesis is markedly elevated when the colon is inflamed (*A*–*C*), whereas fecal H_2S synthesis

remains unchanged (*D*) (**P < 0.01 vs. the corresponding control group). Colonic (*E*), but not fecal (*F*), H_2S synthesis correlates very well with tissue inflammation [as measured by myeloperoxidase (MPO) activity, a marker of granulocyte infiltration]. Each bar represents the mean ± SEM of 4-6 samples.

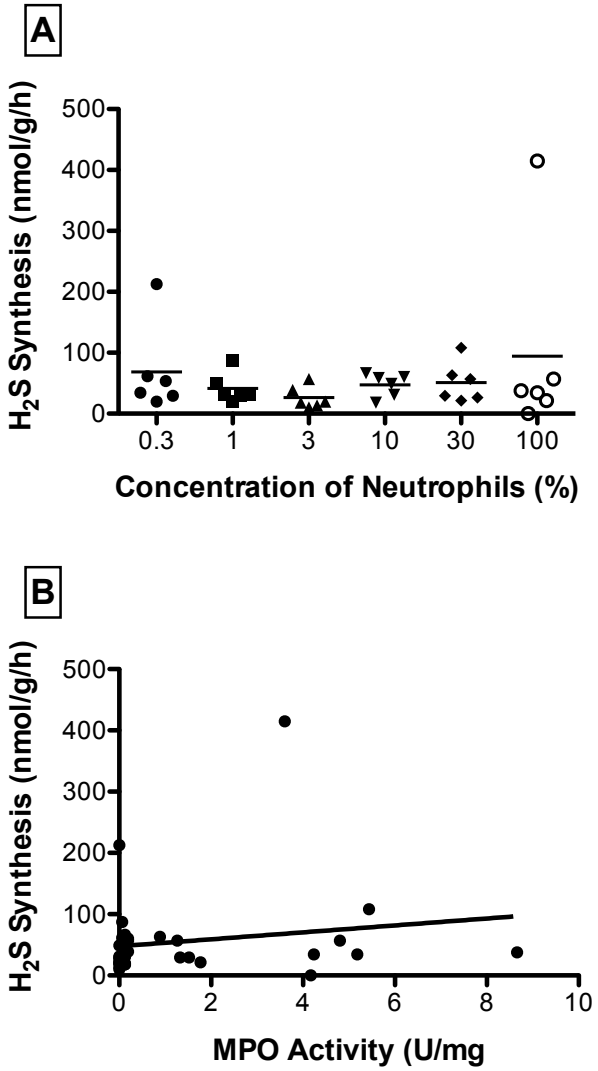

Figure 3.4 – Production of H_2S by various concentrations of neutrophils harvested from the peritoneal cavity of rats. There is no correlation between the concentration of neutrophils (*A*) or the MPO activity (B; R^2 = 0.03; not significant) and the amount of H_2S

produced. There was a highly significant linear correlation between the concentration of neutrophils and MPO activity ($R^2 = 0.98$; $P < 0.001$).

Vitamin B deficiency reduces colonic but not fecal H_2S synthesis

As reported previously (Troen et al., 2008), rats fed a vitamin B-deficient diet for 6 weeks developed significant hyperhomocysteinemia (plasma homocysteine levels of 189 ± 24 vs. 10 ± 2 mol/ L in rats fed the control diet). This was accompanied by an almost complete (95%) reduction of colonic tissue H_2S synthesis compared with controls ($P < 0.05$; Fig. 3.5*A*). However, fecal H_2S synthesis did not differ significantly between the two diets at the beginning or at the end of the 6-week period (Fig. 3.5*B*).

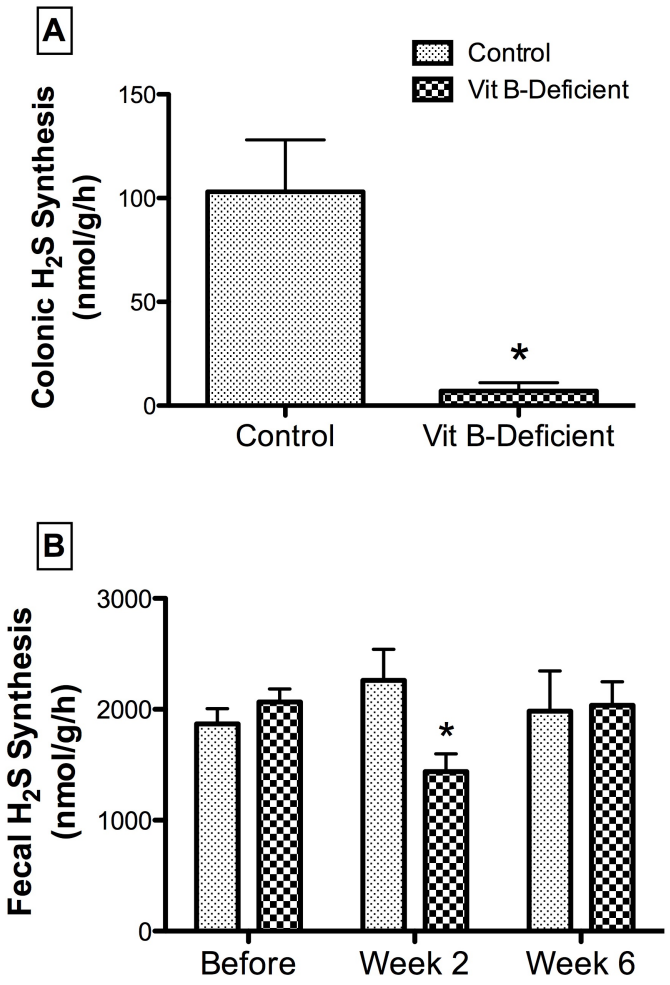

Figure 3.5 – Consumption by rats of a diet deficient in B vitamins results in almost complete suppression of colonic H_2S synthesis but only a small and transitory effect on fecal H_2S synthesis. *Panel A*: Colonic H_2S synthesis after the rats had been on the vitamin B (Vit B)-deficient or control diet (*P < 0.05 vs. the control group). *Panel B*: Fecal H_2S synthesis before and after 2 and 6 weeks of consumption of the vitamin B-deficient or control diets. Fecal H_2S synthesis was significantly (*P < 0.05) decreased after 2 week on the vitamin B-deficient diet but had recovered to control levels by 6 weeks on the vitamin B-deficient diet. Each bar represents the mean ± SEM of 6 samples.

3.4 DISCUSSION

H_2S affects many aspects of digestive function, including blood flow (Zhao et al., 2001), visceral sensitivity (Distrutti et al., 2006; Ekundi-Valentim et al., 2010), smooth muscle contraction (Hosoki et al., 1997; Teague et al., 2002), and secretion (Schicho et al., 2006; Pouokam and Diener, 2011). It also appears to play an important role in regulating inflammatory processes in the gastrointestinal tract (Wallace et al., 2009; Wallace, 2010; Wallace et al., 2012; Zanardo et al., 2006). Thus suppression of H_2S synthesis results in a significant increase in granulocyte numbers in the mucosa, down-regulation of cyclooxygenase-2 (COX-2) expression, and reduced prostaglandin E_2 (PGE_2) synthesis (Wallace et al., 2009). On the other hand, H_2S-donors can accelerate resolution of experimental colitis (Wallace et al., 2009), protect the gastric mucosa from injury induced by non-steroidal anti-inflammatory drugs (Fiorucci et al., 2006; Wallace, 2007; Wallace, 2010; Wallace et al., 2010), and accelerate the healing of gastric ulcers (Wallace et al., 2007b; Wallace et al., 2010). Previous studies have characterized H_2S production throughout the gastrointestinal tract (Martin et al., 2010), demonstrating that the amounts produced on a per gram basis are somewhat greater in the stomach and small intestine than in the colon. Previous studies have also shown (Wallace et al., 2007; Wallace et al., 2009), as in the present study, that colonic H_2S synthesis is markedly increased when the tissue is inflamed/damaged. In the present study, we observed a significant correlation between tissue MPO activity (a marker of granulocyte infiltration) and tissue H_2S synthesis. However, studies of isolated neutrophils failed to show any

such correlation, suggesting that neutrophils were unlikely to be the cellular source of the elevated H_2S synthesis in the inflamed colon.

Of course, in addition to being produced by gastrointestinal tissue, many species of colonic bacteria can also produce H_2S. While colonocytes may act as a "metabolic barrier" to diffusion of luminally derived H_2S in the subepithelial compartment (by efficiently catabolizing H_2S) (Goubern et al., 2007; Lagoutte et al., 2010), it remains possible that some H_2S produced by bacteria could significantly affect colonic function. Moreover, it has been suggested that sulfate-reducing bacteria within the lumen of the intestine could contribute to what is measured as tissue production of H_2S (Linden et al., 2010). This possibility was examined in the present study, and our results strongly suggest that there is no significant contribution of colonic bacteria to the H_2S synthesis measured in samples of colonic tissue. The main evidence for this conclusion is the following: *1*) colonic H_2S synthesis did not differ significantly between germ-free and colonized mice; *2*) H_2S production by inflamed colonic tissue increased proportionately with the severity of inflammation (as measured by MPO activity), but fecal H_2S production remained unchanged; and *3*) feeding rats a vitamin B-deficient diet for 6 weeks almost completely abolished colonic H_2S synthesis but had no effect on fecal H_2S synthesis. Interestingly, rats on the vitamin B-deficient diet displayed significantly reduced fecal H_2S synthesis after 2 weeks on the diet, which is consistent with a portion of fecal H_2S synthesis (i.e., the eukaryotic portion) being vitamin B_6 (P5P) dependent. The observation that, by the end of 6 weeks on the vitamin B-deficient diet, the fecal H_2S

production had returned to the same levels as in controls may suggest changes in the microflora of these rats or in the production of H_2S via alternative pathways by colonic bacteria.

Escherichia coli, *Salmonella enterica*, *Clostridia*, and *Enterobacter aerogenes*, all of which can be found in the large intestine, are among the bacteria that can metabolize cysteine to H_2S (Blachier et al., 2010). H_2S is also produced in the colon by sulfate-reducing bacteria that have the ability to reduce sulfate and other sulfur-containing compounds to H_2S (Blachier et al., 2010). Based on studies in which P5P was excluded from the reaction mixture in the H_2S assay, it would appear that fecal H_2S synthesis is derived ~50% from bacteria and ~50% from eukaryotic cells. This is consistent with bacterial cells comprising about one-half of fecal mass (Tannock, 2007). Colonic (eukaryotic) H_2S synthesis is almost entirely dependent on P5P. Fecal H_2S synthesis was reduced by 50% in the absence of P5P, likely corresponding to inhibition of the eukaryotic component. The observation that fecal samples from germ-free mice produced only about one-half as much H_2S as fecal samples from colonized mice supports this conclusion.

H_2S is a highly reactive gaseous mediator that can freely diffuse across membranes (Mathal et al., 2009). It is rapidly metabolized by the mitochondrial enzyme sulfide quinone reductase (SQR) (Lagoutte et al., 2010) and can be used as an alternative to oxygen in mitochondrial respiration (Goubern et al., 2007; Lagoutte et al., 2010). Colonocytes appear to be particularly well adapted to using H_2S as a metabolic fuel

(Goubern et al., 2007, Li et al., 2011). H_2S can also be sequestered, such as through binding to heme proteins (Li et al., 2011). Thus, measurement of tissue or plasma levels of H_2S in vivo has proven to be very challenging. Unlike the case for nitric oxide, another gaseous mediator, no suitable biomarkers for H_2S have been identified (as nitrate/nitrite are for nitric oxide) (Li et al., 2011). This presents significant challenges in terms of identification of physiological and pathophysiological roles for this gas (Li et al., 2011).

The most commonly employed assay for measurement of tissue production of H_2S is the zinc acetate-trapping technique first described by Stipanuk and Beck (1982). This assay has been used for determination of H_2S synthesis in liver and kidney, gastrointestinal tissues, brain, serum, vascular tissues, and pancreas (Li et al., 2011). The assay may be viewed as a measure of the capacity of a tissue to produce H_2S, since, in most cases, very little H_2S can be detected in the absence of added substrate (L-cysteine). Linden et al. (2010) recently described an elegant method for measuring tissue H_2S production in vitro in the absence of exogenous substrate.

In recent years, there has been considerable evidence emerging for important roles of H_2S in many aspects of digestive health and disease. Tissue production of H_2S appears to be important for maintenance of mucosal integrity, for modulation of inflammation, and for promotion of tissue repair (Fiorucci et al., 2006; Wallace et al., 2007b; Wallace, 2010; Wallace et al., 2010). The fact that H_2S can be used as a metabolic fuel, particularly by colonocytes, further underscores its potential importance in health and disease. The extent to which bacteria-derived H_2S can modulate mucosal function

remains unclear, given the ability of the epithelium to rapidly catabolize this gaseous mediator. On the other hand, in circumstances of epithelial damage or dysfunction, it is possible that the metabolic barrier to diffusion of H_2S in the subepithelial compartment is impaired. The present study provides evidence that, using the most widely applied assay for tissue H_2S synthesis, bacteria do not make a significant contribution to what is measured as colonic H_2S. Colonic H_2S synthesis is markedly up-regulated when the tissue is damaged/inflamed and appears to contribute significantly to resolution and repair.

4.0 ALTERATIONS IN HYDROGEN SULFIDE SYNTHESIS AND DEGRADATION DURING EXPERIMENTAL COLITIS

4.1 INTRODUCTION

Hydrogen sulfide (H_2S) is produced in virtually every organ system in the body (Wang, 2012), and can modulate a variety of physiological processes including vasodilation (Zhao et al., 2001), nociception (Distrutti et al., 2006; Ekundi-Valentim et al., 2010) and inflammation (Dufton et al., 2012; Wallace et al., 2009; Wallace 2010; Zanardo et al., 2006). The importance of H_2S in the gastrointestinal tract is highlighted by its ability to regulate intestinal smooth muscle function (Hosoki et al., 1997; Teague et al., 2002) and epithelial secretion (Schicho et al., 2006; Pouokam and Diener, 2011). H_2S can also enhance ulcer healing (Wallace et al., 2007b; Wallace et al., 2010) and promote resolution of colitis (Wallace et al., 2009). In recent years it has become clear that H_2S is also an important substrate for mitochondrial respiration, driving the production of adenosine triphosphate (ATP) (Goubern et al., 2007; Lagoutte et al., 2010). Colonocytes are particularly well adapted for using H_2S as an energy source (Goubern et al., 2007; Lagoutte et al., 2010). There is also evidence that in hypoxia, mitochondrial H_2S metabolism may underlie the ability of this gaseous mediator to reduce reactive oxygen metabolite-mediated tissue injury (Elrod et al., 2007; Kimura et al., 2010). Such effects may contribute to the ability of H_2S to protect the gastrointestinal mucosa from injury and to promote healing (Elrod et al., 2007; Mard et al., 2012; Wallace et al., 2012).

The majority of H_2S production in mammalian tissue is enzymatically regulated with the pyridoxal-5'-phosphate (P5P)-dependent enzymes cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE) receiving the most focus. During a bout of hapten-induced colitis, the capacity of rat colonic tissue to produce H_2S is markedly increased, in parallel with the degree of mucosal inflammation (Wallace et al., 2009). The H_2S produced in this setting helps drive the resolution of inflammation and enhances the healing of ulcers. Based on pharmacological studies (i.e., using inhibitors of CBS and CSE) CBS appeared to be responsible for the majority of the observed increase in colonic H_2S synthesis (Wallace et al., 2009). However, administration of inhibitors of both CBS and CSE did not completely abolish colonic H_2S production, raising the possibility that a source of H_2S other than CBS and CSE may exist in the colon (Wallace et al., 2009).

Indeed, a previous report examining the brains of CBS knockout (KO) mice showed the presence of another pathway for H_2S synthesis that, unlike CBS and CSE, functioned in the absence of P5P (Shibuya et al., 2009a). Like CBS and CSE, this pathway utilizes L-cysteine as a substrate for H_2S production; however, it involves the enzymes cysteine aminotransferase (CAT) and mercaptopyruvate sulfurtransferase (3MST) (Shibuya et al., 2009a; Shibuya et al., 2009b). CAT, which requires α-ketoglutarate as a cofactor, converts L-cysteine into 3-mercaptopyruvate (3MP), which is then converted by 3MST into H_2S and pyruvate. To date, this pathway has been shown to contribute to H_2S synthesis in the brain and the vascular endothelium of the thoracic aorta (Shibuya et

al., 2009a; Shibuya et al., 2009b). However, the importance of this pathway and its contributions to H_2S synthesis in the gastrointestinal tract are unknown.

In the present study, we examined the contribution of the CAT-3MST pathway to H_2S synthesis in the healthy and inflamed colon. We also attempted to determine the relative contributions of the compartments of the colonic tissue (mucosa versus muscularis) to H_2S synthesis. Finally, tissues were examined for their H_2S-inactivating capacity.

4.2 METHODS AND MATERIALS

Animals

Male, Wistar rats were housed in plastic cages and maintained under controlled temperature (20°C), humidity (60%-70%), and light cycle (12 h:12 h light-dark). The rats were fed standard laboratory chow and water *ad libitum*. All experimental protocols were approved by the Animal Research Ethics Board at McMaster University, and adhered to the guidelines established by the Canadian Council on Animal Care.

The colonic tissue from CSE knockout (KO) mice and from age-matched (16 weeks), wild type (WT) littermates on the C57BL/6J x 129SvEv background were generously provided by Dr. Rui Wang (Lakehead University, Canada). These mice were generated by targeted disruption of the CSE gene (Yang et al., 2008).

Induction of Colitis

Colitis was induced in conventionally housed rats using dinitrobenzene sulfonic acid (DNBS) (Wallace et al., 1995). Rats were intrarectally administered 0.5 mL of 60 mg/mL DNBS in 50% ethanol. Groups of rats (n = 4 – 6) were euthanized 6 hours to 28 days after TNBS administration for determination of H_2S synthesis. Colonic inflammation was assessed by the measurement of myeloperoxidase (MPO) activity, as first described by Bradley et al. in 1982. MPO is an enzyme found primarily in the azurophilic granules of neutrophils, and has been used extensively as a quantitative index of granulocyte infiltration (Bradley et al., 1982; Wallace et al., 1990). In subsequent colitis studies, rats were euthanized on day 3 when colitis was determined to be most robust.

Separation of Colonic Layers

After the rats were euthanized, the colons were excised, opened along the mesenteric boarder, washed thoroughly to remove fecal material, and immediately placed in ice-cold potassium phosphate buffer (pH 8.0; 12% w/v). Sections of the distal colon were visualized under a dissecting microscope and tightly pinned along the edges of the sections with care taken to avoid causing damage to the tissue. The border between the mucosa and muscularis was identified, then these two layers were gently separated using forceps and immediately snap-frozen in liquid nitrogen. In rats that had received DNBS, the mucosal and muscularis were separated, then sections were cut at the border between the ulcer and the macroscopically normal tissue, yielding separate

sections of mucosa and muscularis from both ulcerated and non-ulcerated areas. Sections of each layer were fixed in 10% formalin and later stained with hemotoxilyn & eosin (H&E) for microscopic examination to confirm separation of the mucosal layer from the muscularis.

H_2S Synthesis

The capacity of tissue to produce H_2S was measured from homogenized tissue in the presence of exogenous substrate and/or inhibitors using a modified version of a previously described zinc-trapping assay (Stipanuk and Beck, 1982; Wallace et al., 2007b). Production of H_2S via the CAT-3MST pathway was determined using the substrate α-ketoglutarate and the CAT inhibitors L-aspartate (a competitive inhibitor of CAT) and O-carboxymethyl-hydroxylamine hemihydrochloride (CHH; an inhibitor of aminotransferases, including CAT). All reactions required exogenous L-cysteine for H_2S synthesis to occur. 10 mM L-cysteine was used in all reactions. H_2S production via CBS and CSE, also required the presence of pyroxidal-5'-phosphate (P5P).

Isolation of rat peritoneal neutrophils

To examine the capacity of neutrophils to produce H_2S, activated neutrophils were collected from the rat peritoneum as previously described (Boughton-Smith et al., 1988). Peritonitis was induced using 20 mL of a 1% solution of glycogen (from oyster, type II) injected intraperitoneally (20-G needle). Peritoneal fluid was collected 4 hours

after. The peritoneal fluid was centrifuged (15 x g, 15 min), and the resulting pellet was resuspended in 10 mL of heparinized saline (25 U/ mL). Further dilutions of these cells were made in isotonic saline. These various concentrations of cells were assayed for MPO activity and H_2S synthesis following the same procedures as described above.

H_2S-inactivation

The ability of tissue to metabolize and/or sequester H_2S was measured using a modified version of the above-mentioned zinc-trapping assay. Instead of including L-cysteine as a substrate for H_2S synthesis, vials containing either tissue homogenates or buffer were 'spiked' with 33 µL of 30 µM NaHS. The samples were then incubated for 5-90 min in a 37°C shaking water bath, after which trichloroacetic acid (TCA; 50%; 0.4 mL) was injected into the reaction mixture through the serum cap. The vials were then transferred to a 50°C shaking water bath for 60 min to allow for the trapping of evolved H_2S by the zinc acetate. The greater the ability of a tissue to metabolize/sequester H_2S, the less H_2S would be trapped in the zinc acetate. To determine the maximal recovery of H_2S from this system, samples were treated in the same way, except that the TCA was added prior to addition of the NaHS.

4.3 RESULTS

Healthy colonic tissue produces H_2S via the CAT-3MST pathway

The healthy colon produced H_2S in the presence of α-ketoglutarate and the absence of P5P, consistent with H_2S synthesis via the CAT-3MST pathway (Figure 4.1). H_2S synthesis by the healthy colon increased in response to increasing concentrations of α-ketoglutarate (Figure 4.1*A*). Maximal H_2S synthesis was observed with 300 μM α-ketoglutarate. Unless otherwise noted, 100 μM of α-ketoglutarate was added to colonic tissue (healthy and inflamed) in subsequent experiments.

When H_2S synthesis was measured in the healthy colon in the presence of α-ketoglutarate and a CAT inhibitor (L-aspartate or CHH), H_2S synthesis was significantly inhibited in a dose-dependent manner (Figure 4.1, *B & C*).

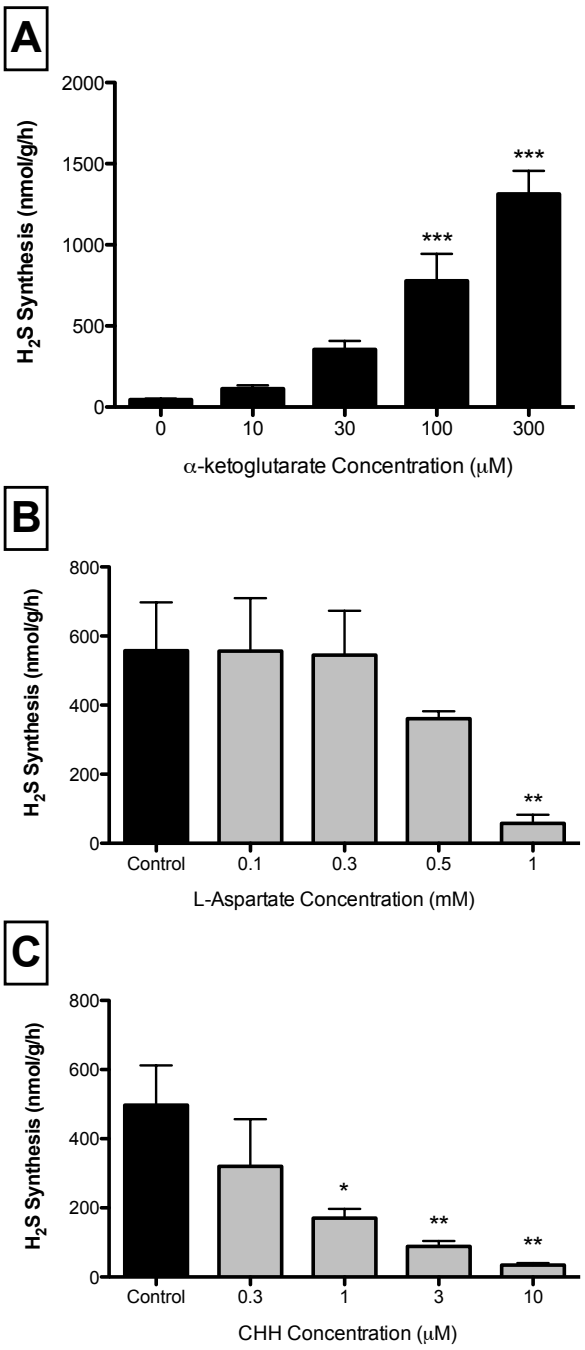

Figure 4.1 – H_2S synthesis by the healthy colon in the presence of varying concentrations of α-ketoglutarate and inhibitors of CAT. α-ketoglutarate increased the production of H_2S in the colon in a concentration-dependent manner (***P < 0.001 vs.

controls with no α-ketoglutarate added). At a concentration of 1 mM, L-aspartate significantly suppressed H_2S synthesis by healthy colonic tissue (*Panel A*). Micromolar concentrations of CHH were able to inhibit H_2S production (*Panel B*) (*P < 0.05, **P < 0.01 vs. corresponding control groups). Each bar represents the mean ± SEM of 4-6 samples.

CSE is the major contributor to P5P-dependent H_2S synthesis in the healthy colon

Colonic P5P-dependent H_2S synthesis was almost completely absent in mice deficient for the CSE enzyme (Figure 4.2). Furthermore, production of H_2S in the healthy colon occurred in the absence of P5P and the presence of α-ketoglutarate (i.e., through the CAT-3MST pathway). However, the ability of the colon from CSE KO mice to produce H_2S via the CAT-3MST pathway was also significantly reduced (by ~65%) in comparison to that in WT mice.

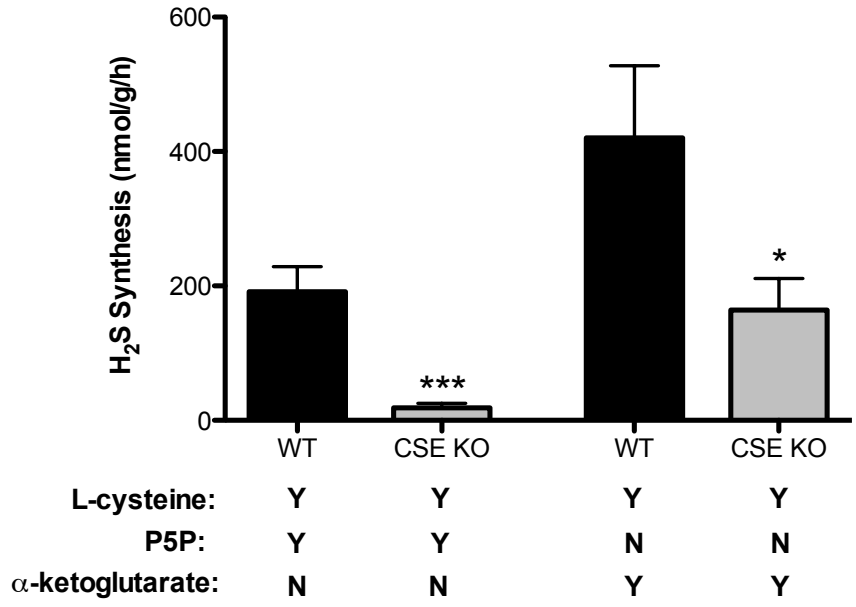

Figure 4.2 – Colonic H_2S synthesis in CSE knockout (KO) mice vs. wild-type (WT) mice via CBS/ CSE and CAT-3MST pathways. The presence or absence of L-cysteine, P5P or α-ketoglutarate is indicated by "Y" and "N", respectively. In the presence of P5P, colonic tissue from CSE-deficient mice produced significantly less H_2S from L-cysteine than colonic tissue from WT mice (***$P < 0.001$ vs. corresponding WT mice). The ability of colonic tissue from CSE KO mice to produce H_2S from L-cysteine in the presence of α-ketoglutarate (via CAT-3MST) was also markedly decreased when compared to colonic tissue from WT mice (*$P < 0.05$ vs. corresponding WT control mice). Each bar represents the mean ± SEM of 7 samples.

Colonic H_2S is produced via CAT-3MST during experimental colitis

H_2S synthesis over the time-course of DNBS colitis was measured in colonic homogenates in the presence of L-cysteine (10 mM) and α-ketoglutarate (100 μM). During inflammation the capacity of the colon to produce H_2S via CAT-3MST was markedly increased (Figure 4.3). The greatest H_2S synthesis occurred on day 3 when inflammation was most robust, as indicated by MPO activity (Figure 4.3*A*). Production of colonic H_2S via CAT-3MST returned to control levels by day 28, when inflammation was almost completely resolved (Figure 4.3). Furthermore, the increased levels of colonic H_2S synthesis observed 3 days after the induction of colitis were suppressed by both L-aspartate and CHH (Figure 4.4), consistent with this synthesis occurring via the CAT-3MST pathway.

Colonic MPO activity and H_2S synthesis both peaked at day 3 (Figure 4.3). This observation raised the possibility that neutrophils could be a primary source of H_2S synthesis via the CAT-3MST pathway. To explore this, we measured H_2S synthesis by neutrophils harvested from the peritoneum of rats (following i.p. injection of oyster glycogen) in the presence of L-cysteine (10 mM) and α-ketoglutarate (100 μM). Neutrophils were not capable of producing H_2S via CAT-3MST as indicated by negligible levels of H_2S when measured in the presence with α-ketoglutarate (data not shown).

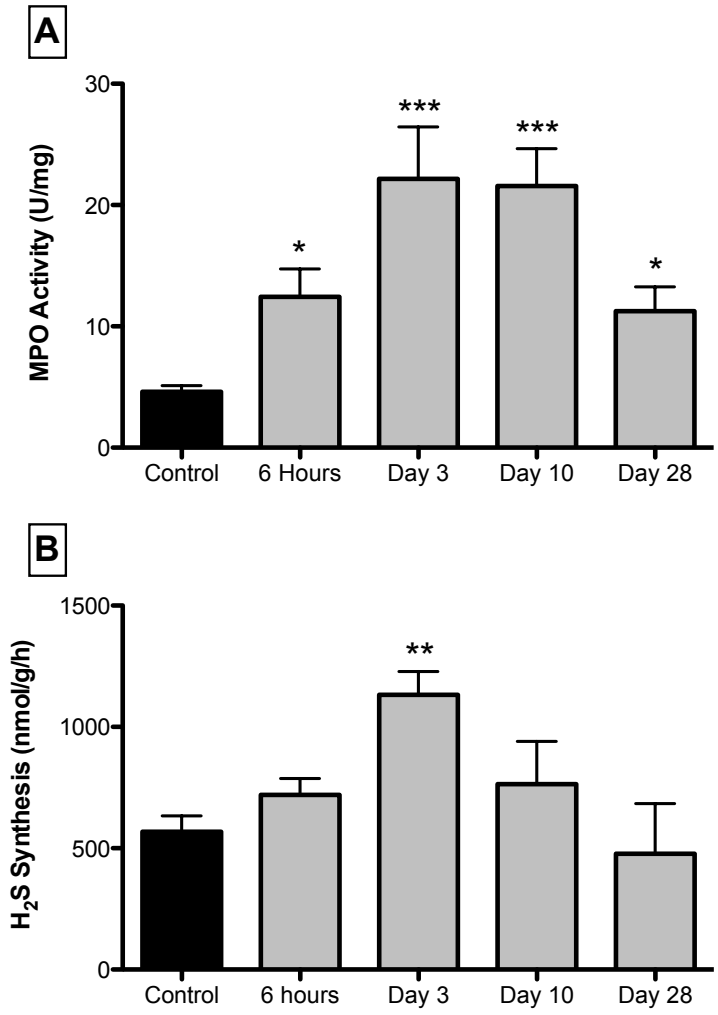

Figure 4.3 – Hydrogen sulfide synthesis via CAT-3MST over the course of colitis induced by DNBS. *Panel A*: The severity of colitis at different time points as measured by MPO, a biochemical marker of granulocyte infiltration (*P < 0.05, ***P < 0.001 vs. controls). *Panel B*: Production of H_2S over the course of colitis. Colonic samples were incubated with L-cysteine (10 mM) and α-ketoglutarate (100 μM). A significant increase in colonic H_2S synthesis was observed on day 3 after DNBS instillation (**P < 0.01 vs. controls). Each bar represents the mean ± SEM of 4-6 samples.

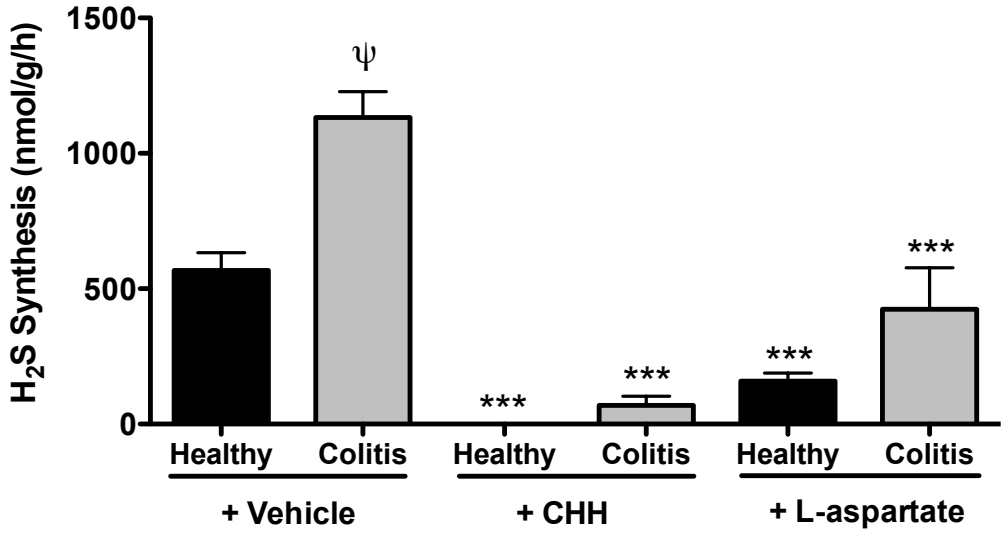

Figure 4.4 – Inhibition of colonic H_2S synthesis by CAT inhibitors. H_2S synthesis via CAT-3MST markedly increased in the inflamed colon (taken 3 days after DNBS administration) (ψ $P < 0.001$ vs. corresponding healthy control). L-aspartate (1mM) and CHH (10 μM) significantly suppressed H_2S synthesis by samples of inflamed colon (***$P < 0.001$ vs. corresponding colitis groups in the absence of inhibitors). Each bar represents the mean ± SEM of 4-6 samples.

CAT-3MST is the main pathway for H_2S production in the healthy and inflamed colon

Having established the presence of the CAT-3MST pathway in the colon, we examined the importance of this pathway by comparing the optimal contributions of the CBS/ CSE and CAT-3MST pathways to colonic H_2S synthesis. To determine the relative contributions of the CBS/ CSE versus the CAT-3MST pathways to colonic H_2S synthesis, we determined the optimal concentrations of co-factors (P5P and α-ketoglutarate, respectively) for each pathway, by performing concentration-response studies. Maximal production of H_2S via the CBS/ CSE pathways was observed with P5P at 3 mM, while maximal production of H_2S via the CAT-3MST pathway was observed with α-ketoglutarate at 300 μM. As shown in figure 2*B*, under these optimized conditions, the CAT-3MST pathway accounted for about 6-times as much colonic H_2S synthesis as that produced via the CBS/ CSE pathways ($P < 0.001$).

The contributions of the CBS/ CSE and CAT-3MST pathways were also examined in the inflamed colon; however, the concentrations of P5P (2 mM) and α-ketoglutarate (100 μM) were adjusted (Figure 4.6). In concordance with a previous report (Wallace et al., 2009), we found that H_2S production by inflamed colonic tissue markedly increased when incubated with P5P, when compared to healthy colonic tissue ($P < 0.05$). However, when inflamed colonic tissue was incubated in the presence of α-ketoglutarate, H_2S synthesis from L-cysteine was > 3-times that contributed by the CBS/ CSE pathway ($P < 0.05$).

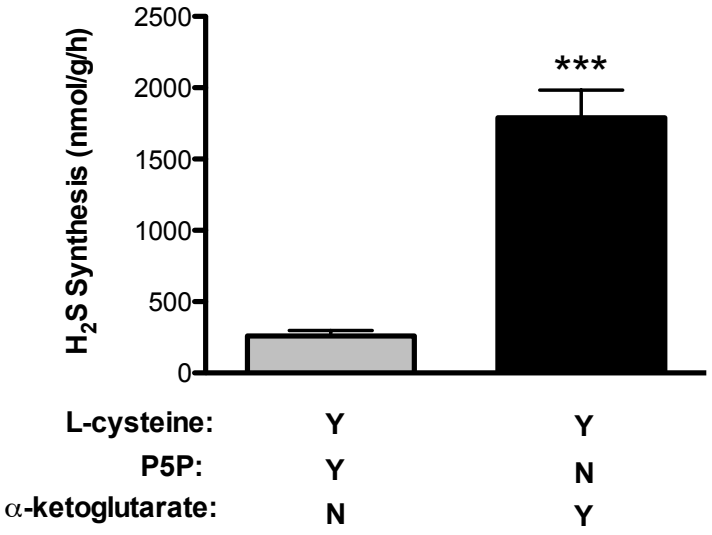

Figure 4.5 – Contributions to H_2S synthesis from the CBS/ CSE or CAT-3MST pathways in healthy colonic tissue. Maximal production of H_2S from L-cysteine by the healthy colon was observed in the presence of 3 mM P5P or 300 μM α-ketoglutarate. The maximal production of H_2S in the healthy colon in the presence of α-ketoglutarate was markedly higher than the maximal production of H_2S in the presence of P5P (***P < 0.001). Each bar represents the mean ± SEM of 5 samples.

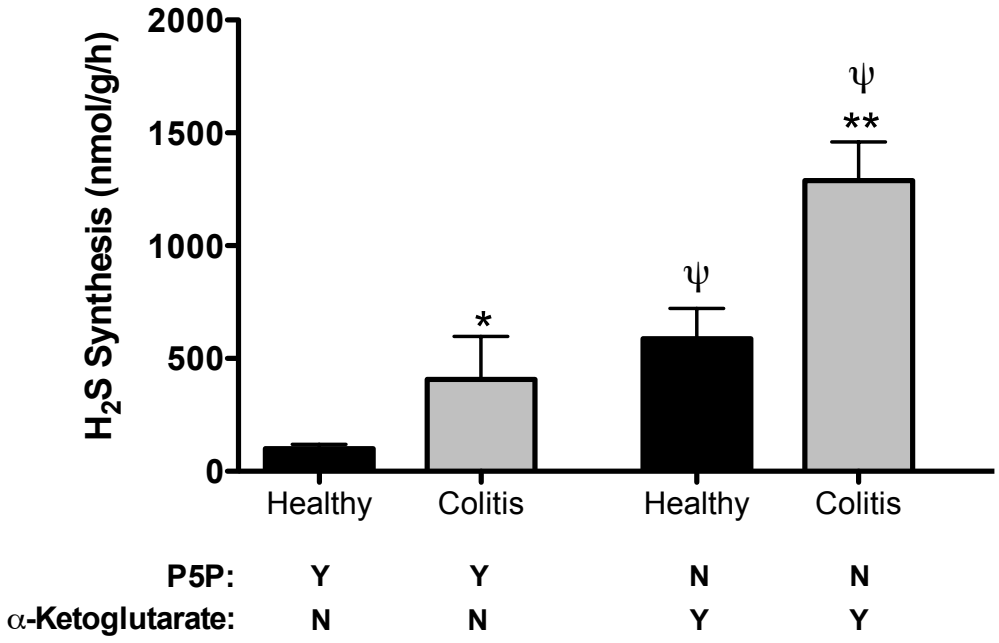

Figure 4.6 – H_2S synthesis from the healthy and inflamed rat colon in the presence of various combinations of substrates and co-factors. The ability of the inflamed colon (taken from rats 3 days after DNBS administration) to produce H_2S from L-cysteine markedly increased in the presence of P5P (*P < 0.05 vs. corresponding healthy group). H_2S production from L-cysteine in the presence of α-ketoglutarate and absence of P5P (through CAT-3MST) also markedly increased in the inflamed colon (**P < 0.05 vs. corresponding healthy group). Levels of H_2S produced via CAT-3MST in the inflamed colon were significantly higher than those produced in the inflamed colon via CBS/ CSE. (ψ P < 0.05 colitic tissue in the presence of α-ketoglutarate but the absence of P5P vs. colitic tissue in the presence of P5P but the absence of α-ketoglutarate). Each bar represents the mean ± SEM of 4-6 samples.

The ulcerated mucosa is the major site of H_2S production during colitis

H_2S synthesis via CAT-3MST from the mucosa and muscularis layers of the colonic tissue from healthy rats was comparable (Figure 7*A*), as was the case for H_2S synthesis via P5P-dependent pathways (Fig. 7*B*). However, the mucosa from sites of ulceration (3 days after DNBS) produced significantly more H_2S via the CAT-3MST pathway (Fig. 7*A*) and the P5P-dependent pathways (Fig. 7*B*). H_2S synthesis from samples of the muscularis layer were relatively low, though a significant increase in synthesis via the P5P-dependent pathways was observed when the tissue was from a region of ulceration (Fig. 7*B*). H_2S synthesis from mucosal or muscularis samples from rats with colitis, but taken from sites that were not ulcerated, was similar to that from samples taken from healthy controls. However, the elevated synthesis of H_2S from tissues taken from sites of ulceration did not appear to be related to inflammation at those sites. MPO activity, a marker of granulocyte infiltration, was comparable in samples of mucosa from sites of ulceration versus non-ulcerated sites (21.5 ± 0.5 U/mg and 17.8 ± 2.0 U/mg, respectively; ns). The same was the case for samples of muscularis from sites of ulceration versus non-ulcerated sites (10.9 ± 2.9 U/mg vs. 8.6 ± 3.2 U/mg, respectively; ns). Thus, both the mucosa and muscularis samples displayed marked inflammation, in contrast to samples from healthy controls (MPO values of 5.1 ± 0.5 U/mg for mucosa and 0.5 ± 0.2 U/mg for muscularis). The inflammation of mucosal and muscularis samples from sites of ulceration was confirmed by histology.

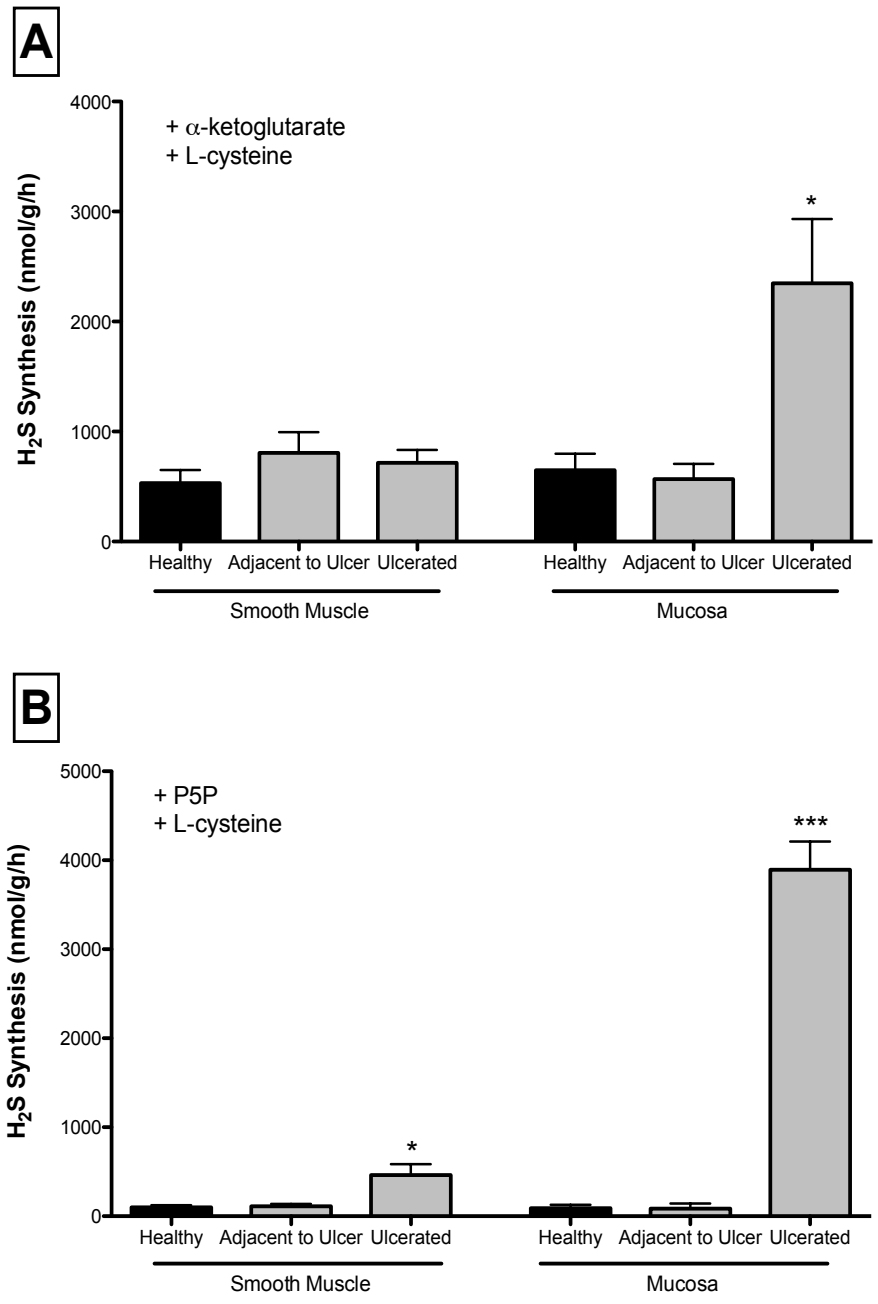

Figure 4.7 – H_2S synthesis from compartments of colonic tissue (mucosa vs. muscularis) of the healthy and inflamed colon. *Panel A*: The contribution of the mucosa and muscularis to H_2S synthesis via the CAT-3MST pathway. H_2S synthesis by the ulcerated mucosa was markedly increased when compared to that of the healthy mucosa (*P <

0.05 vs. healthy mucosa). *Panel B*: Contributions to colonic H_2S synthesis from the CBS/ CSE pathways. H_2S production by ulcerated muscularis and ulcerated mucosa increased significantly when compared to healthy muscularis and healthy mucosa, respectively (*P < 0.05 vs. healthy smooth muscle, ***P < 0.001 vs. healthy mucosa). Increased H_2S production from all pathways during ulceration occurred only at the site of ulceration and not at sites adjacent to ulcers. Each bar represents the mean ± SEM of 4-6 samples.

Colonic H_2S degradation is impaired during ulceration

Both the healthy and inflamed colon rapidly inactivated H_2S *in vitro* (Figure 8*A*). Most inactivation of H_2S occurred within the first 30 min, but there was significantly less inactivation of H_2S in samples from rats with colitis than in healthy controls. This difference was still apparent after 60 min of incubation of tissues, but by 90 min there was no significance between the groups. Approximately 20% of the H_2S that was added to the samples could not be recovered, and presumably was bound to tissue. Indeed, pretreatment of the tissue samples with potassium cyanide (1 mM) increased the recovery of H_2S after 30 min of incubation substantially (to 56.7 ± 5.6% for tissue from healthy rats, and 63.6 ± 4.6% for tissue from rats with colitis). This suggests that the majority of the decrease in recoverable H_2S from the tissue samples was due to metabolic inactivation of H_2S.

We further examined the ability of the inflamed colon to inactivate H_2S by examining sections of the mucosa and muscularis from healthy rats and rats with colitis.

Recovery of H_2S from mucosal samples taken from sites of ulceration was markedly less than that from non-ulcerated sites or from samples from healthy controls (Figure 8*B*). No differences in recovery of added H_2S were observed for muscularis samples from healthy rats or rats with colitis, and there were no differences in H_2S recovery between samples taken from ulcerated versus non-ulcerated sites (data not shown).

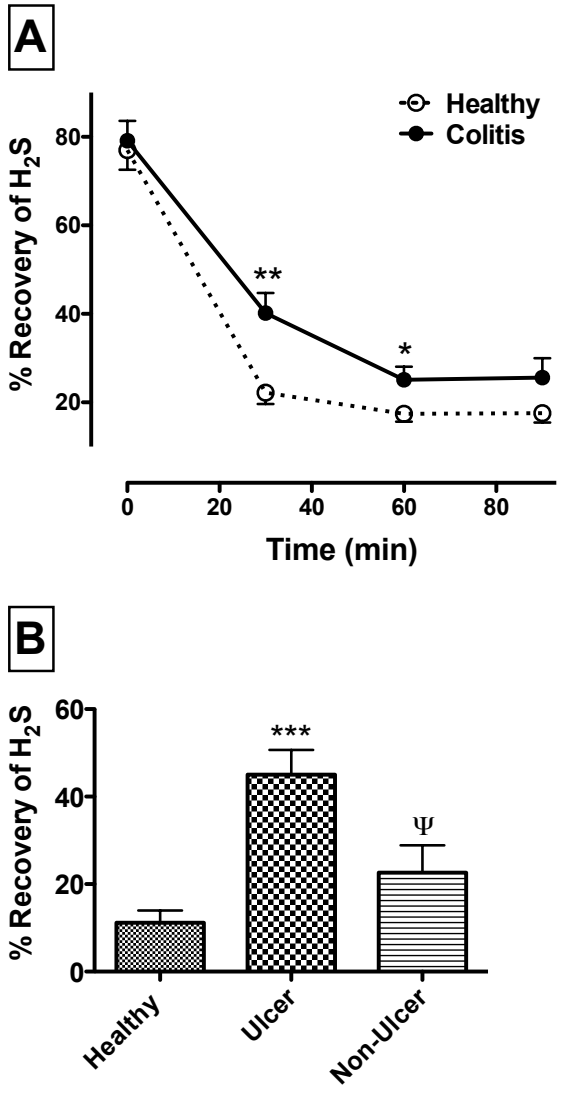

Figure 4.8 – Catabolism of H_2S by healthy and inflamed colonic tissue. *Panel A*: Catabolism of H_2S was significantly reduced in tissue from rats with colitis as compared to that in tissue from healthy controls (*$p<0.05$, **$p<0.01$). Each point represents the mean ± SEM of 5-10 samples. *Panel B*: Catabolism of H_2S was significantly reduced in mucosal tissue from sites of ulceration as compared to mucosal tissue from healthy

controls (***$p<0.001$) and as compared to mucosal tissue from non-ulcerated sites in rats with colitis ($^{\psi}p<0.05$). Each bar represents the mean ± SEM of 5 samples.

4.4 DISCUSSION

H_2S is produced throughout the gastrointestinal tract and contributes to many vital digestive functions, including epithelial secretion (Ise et al., 2011), smooth muscle contraction (Hosoki et al., 1997) and mucosal defense (Blackler et al., 2012; Fiorucci et al., 2005; Wallace, 2010). H_2S is also important for promoting resolution of inflammation and repair of injury (Wallace et al., 2007b; Wallace et al., 2009; Wallace et al., 2010; Wallace et al., 2012). Both of these effects have been demonstrated in animal models of gastrointestinal inflammation and ulceration (Wallace et al., 2007b; Wallace et al., 2009), in which H_2S synthesis is markedly increased. In the present study, we have demonstrated that, in contrast to what has been reported previously (including by ourselves), colonic enzymatic H_2S synthesis does not occur solely through P5P-dependent pathways (CBS and CSE). Rather, a P5P-independent pathway, via the enzymes CAT and 3MST, is a major source of H_2S synthesis in both the healthy and inflamed colon. We also demonstrated that the ulcerated mucosa is the major site of both P5P–dependent and –independent H_2S synthesis and that this synthesis is not influenced by the extent of granulocyte infiltration into the tissue. Furthermore, inactivation of H_2S (via catabolism and/or sequestration) occurs rapidly in colonic tissue

(mucosa more so than muscle), but at a lower rate in inflamed colonic tissue from rats with colitis than tissue from healthy rats.

Currently, little is understood about the contributions of the CAT-3MST pathway to H_2S synthesis in mammalian tissues. This pathway for P5P-independent H_2S synthesis, although identified in the brain and vasculature of rats, has yet to be identified in the gastrointestinal tract. In the present study, we have shown through the use of H_2S synthesis measurement assays and inhibitors of H_2S synthesis that the CAT-3MST pathway contributes to H_2S synthesis in both the healthy and inflamed colon. Importantly, the CAT-3MST pathway made greater contributions to colonic H_2S synthesis than the previously studied pathways (CBS and CSE), in the healthy and inflamed colon (Figure 4.5 and 4.6). The highest levels of colonic H_2S synthesis via CAT-3MST were observed when inflammation was most robust (3 days post DNBS). By day 28, when colitis had largely resolved, H_2S synthesis decreased to control levels (Figure 4.3). Previous reports have shown that exogenous and endogenous sources of H_2S can drive the resolution of inflammation (Wallace et al., 2009). Based on the observation that the CAT-3MST pathway was a greater contributor to H_2S synthesis in the inflamed colon than the CBS/ CSE pathways, it may actually be the role of this 3rd pathway for H_2S synthesis to resolve inflammation. The use of H_2S inhibitors may help find answers to this question, however no inhibitors of 3-MST mediated H_2S production have been identified.

While examining the pathways for H_2S synthesis we found that H_2S production from the CAT-3MST pathway was inhibited by CHH, an inhibitor of CAT (Cornell, 1984; Whiteman and Winyard, 2011). CHH has also been widely used as an inhibitor of CBS and has been shown to suppress H_2S synthesis in the presence of P5P (Fiorucci et al., 2005; Whiteman and Winyard, 2011). It is possible that several studies in the current literature have over-estimated the role of CBS in many physiological settings *in vivo*, as CHH also inhibits H_2S production via the CAT-3MST pathway. This has also limited the ability to use CHH to help distinguish the roles of the different H_2S producing enzymes *in vivo*.

With the knowledge that the most commonly used H_2S synthesis inhibitors including CHH are non-selective, we decided to examine mice genetically deficient for CSE to gain a better understanding of P5P-dependent H_2S synthesis in the colon. Studies have been performed in mice genetically deficient in CSE mice with regards to the cardiovascular system (Yang et al., 2008), however no studies to date have been published regarding the gastrointestinal phenotypes of these mice. Here we demonstrate the first studies examining colonic H_2S production in CSE knock out (KO) mice. In healthy colons from CSE KO mice, H_2S production in the presence of P5P was almost completely absent when compared to WT controls (Figure 4.2). These data, in contrast to previous work using inhibitors, demonstrated that CSE is the major P5P-dependent enzyme for H_2S production in the healthy colon. Remarkable was the fact that in CSE KO mice the production of H_2S via CAT-3MST was also significantly

attenuated when compared to WT mice. It is possible that the pathways for H_2S synthesis may regulate the expression or function of each other much like the interactions observed between NO-producing and H_2S-producing pathways (Coletta et al., 2012).

When considering the tissue source of H_2S, especially the sources accounting for increased H_2S synthesis during inflammation, the literature is rather disparate. Previous evidence suggests that colonic smooth muscle is a source of H_2S (Linden et al., 2008). On the other hand, staining experiments have found the localization of CBS and CSE to be diffuse throughout the colon of rats (Martin et al., 2010). Activated macrophages are also capable of producing H_2S (Dufton et al., 2012), however the levels of H_2S measured in macrophages (around 2 nmol/ 10^6 cells / hr) are much lower than levels of H_2S found when measuring total colonic H_2S synthesis. Moreover, our current data show that MPO and H_2S levels peak in the colon at the same time during the course of DNBS colitis, raising the possibility that neutrophils may be a significant source of H_2S. However, using activated neutrophils from the rat peritoneum, we found that neutrophils produced negligible levels of H_2S and were not a significant source of H_2S synthesis via both P5P–dependent and –independent pathways (CBS/ CSE and CAT-3MST, respectively). By taking a more gross anatomical approach, we separated the colon into distinct layers of muscularis and mucosa to get a better understanding of H_2S production in these compartments. We observed that the ulcerated mucosa is the major contributor to H_2S synthesis during DNBS-induced colitis. At sites of mucosal ulceration the ability of tissue

to produce H_2S via CAT-3MST is markedly increased when compared to healthy and non-ulcerated mucosa. The levels of H_2S synthesis from the CBS/ CSE pathways were also markedly increased in the ulcerated mucosa when compared to the healthy and non-ulcerated mucosa. Taken together, the ulcerated mucosa accounted for the greatest levels of H_2S synthesis, and the extent of granulocyte infiltration into tissue was not a significant determinant of levels of H_2S production. It is clear that damage to colonic tissue is the major stimulus for increased H_2S synthesis.

Colonocytes of the epithelial barrier contain a robust set of mitochondrial enzymes that possess the ability to oxidize H_2S into thiosulfate, thus inactivating H_2S and its local effects (Goubern et al., 2007; Linden et al., 2012; Mimoun et al., 2012). In fact, colonic epithelial cells are very well adapted to the high luminal concentrations of H_2S in the colon and through mitochondrial activity can "detoxify" H_2S while also driving the production of adenosine triphosphate (ATP) (Goubern et al., 2007; Lagoutte et al., 2010). Sulfide quinone reductase (SQR), an enzyme capable of oxidizing H_2S into thiosulfate is highly expressed in the colonic mucosa (Linden et al., 2012). In the present study we have demonstrated that the colon possesses a robust ability to inactivate H_2S. This was done by measuring the ability of tissue to inactivate H_2S using a variation of the zinc-trapping assay. As previously reported, this process of colonic H_2S-inactivation was rapid, occurring within minutes (Vitvitsky et al., 2012) and within 30 minutes the majority of H_2S had been inactivated. Moreover, inactivation of H_2S was significantly reduced in tissue from rats with colitis. This impaired ability to inactivate H_2S occurred at

the site of the ulcerated mucosa. It is possible that a breach in the epithelial layer resulting from DNBS administration led to a loss of H_2S-inactiviting enzymes, including SQR, in the epithelial layer and thus the ability to inactivate H_2S. Local tissue concentrations of H_2S are influenced by both the rate of enzymatic production and the rate of inactivation of H_2S (Kimura, 2012). Considering the ability of H_2S to enhance ulcer healing, a loss in the ability to breakdown H_2S may be beneficial for allowing increased tissue concentrations of H_2S directly at the site of damage.

In summary, CAT and 3MST represent the primary pathway for H_2S synthesis in the healthy and inflamed colon. These data represent the first report of the presence and function of a novel pathway (CAT-3MST) for the production of H_2S in the gastrointestinal tract. The increased production of H_2S via the CAT-3MST enzymes appears to be occurring at the site of mucosal ulceration. The CBS/ CSE pathways also make important contributions to H_2S synthesis at the ulcerated mucosa. Furthermore, during colitis, the damaged colon exhibits an impaired ability to inactivate H_2S. Increased production of H_2S via multiple enzymatic pathways as well as impaired H_2S-inactivation may contribute to increased levels of H_2S at site of ulceration. Combined, these effects may increase local tissue concentrations of H_2S that can drive the resolution of colitis and the healing of ulcers. A further understanding of the enzymatic and catabolic pathways for the regulation of H_2S production in the colon can present new opportunities for modulating colonic H_2S production, which may have important implications in the development of novel therapies for inflammatory bowel disease.

5.0 GENERAL DISCUSSION

5.1 Conclusions

Like nitric oxide (NO) and carbon monoxide (CO), H_2S has historically been recognized as an industrial pollutant but is now accepted as an important mediator of many physiological and pathophysiological processes (Wallace, 2010). H_2S is known to play fundamental roles in the cardiovascular system as a vasodilator (Zhao et al., 2001) and in the central nervous system as a neuromodulator (Abe and Kimura, 1996). There is now considerable evidence supporting the important role of H_2S in many aspects of digestive health and disease. H_2S can modulate a variety of inflammatory processes including leukocyte trafficking (Zanardo et al., 2006), and the expression and secretion of several pro-inflammatory mediators (Li et al., 2009; Wallace et al., 2009). Furthermore, tissue production of H_2S appears to be important for maintenance of mucosal integrity, the promotion of tissue repair, and the resolution of colitis (Fiorucci et al., 2006; Wallace et al., 2007b; Wallace et al., 2009; Wallace et al., 2010; Wallace, 2010). It is well established that during injury and inflammation in the gastrointestinal tract the ability of tissue to produce H_2S markedly increases (Wallace et al., 2007b; Wallace et al., 2009). In the present thesis we aimed to further examine the production of H_2S in the colon during a bout of colitis.

The production of H_2S occurs in mammalian cells through the enzymatic degradation of L-cysteine. The most widely studied and targeted enzymes for H_2S production are the pyridoxal-5'-phosphate (P5P)-dependent enzymes cystathionine-β-

synthase (CBS) and cystathionine-γ-lyase (CSE). There is also emerging evidence for a 3rd pathway for H_2S synthesis involving the enzymes CAT and 3MST. Finally, another source of H_2S that cannot be forgotten when considering the gastrointestinal tract is the microbiota. This thesis focused on these sources of H_2S with respect to the healthy and inflamed colon. First, we examined the contributions of the microbiota to what is measured as colonic H_2S synthesis. Then we focused on colonic tissue and the importance of the CAT-3MST pathway for H_2S production.

Many of the species of bacteria residing in the gastrointestinal tract are capable of producing H_2S. These include sulfate-reducing bacteria and bacterial species such as *Escherichia coli and Salmonella enterica* (Blachier et al., 2010). The literature concerning the concentrations of H_2S that accumulates in the colonic lumen from bacterial production is disparate. Early studies suggested that the concentrations of H_2S in the lumen of the gut were extremely high relative to levels that occur in the body (Macfarlane et al., 1992; Magee et al., 2000). These levels were estimated to be in the millimolar range (Babidge et al., 1998; Roediger et al., 1993), however it is now understood that luminal concentrations are closer to micromolar levels with most H_2S being bound to fecal material (Levine et al., 1998; Picton et al., 2002).

As mentioned above, colonic production of H_2S is markedly increased when the mucosa is inflamed (Wallace et al., 2009). Because measurements of H_2S in this context are generally performed in vitro, and because of the capacity of some colonic bacteria to produce H_2S, it was possible that bacteria have contributed to what we measured as

"colonic H_2S synthesis''. The first study of this thesis was performed to determine if some portion of what we measure as ''colonic H_2S synthesis'' is actually bacterial H_2S synthesis. Using germ-free mice and mice colonized with altered Schaedler flora (ASF), we measured colonic H_2S synthesis and observed no difference between the two groups. This observation indicated that bacteria (in the colonized mice) were not contributing to H_2S measurements. We also measured tissue and fecal H_2S production from healthy mice and mice with colitis induced by TNBS. Colonic H_2S synthesis markedly increased when the tissue was inflamed. The increased H_2S synthesis correlated with the extent of inflammation as measured by granulocyte infiltration, but neutrophils (the main infiltrating granulocytes) were not a significant source of H_2S synthesis. In addition, fecal H_2S synthesis did not change during colonic inflammation. Lastly, when fed a B vitamin-deficient diet for 6 weeks rats exhibited diminished colonic H_2S synthesis. However fecal H_2S synthesis was not different from that of rats on the control diet, suggesting what we measured as colonic H_2S was coming from colonic tissue itself. Taken together, these studies clearly demonstrated that the H_2S synthesis measured using the *in vitro* zinc-trapping method was not from bacteria adherent to the tissue samples and was instead derived from the colonic tissue.

We next looked at eukaryotic contributions to colonic H_2S synthesis to gain a better understanding of the production of H_2S in terms of its enzymatic production, and metabolism. Previous reports have suggested that increased levels of H_2S production during colonic inflammation are derived from the P5P-dependent enzymes CBS and CSE

(Wallace et al., 2012). CBS appeared to be the main contributor to H_2S levels in the colon, with CSE also making important contributions (Wallace et al., 2009). However, inhibitors of CBS and CSE were not able to completely abolish H_2S production during inflammation, suggesting another source of H_2S in the colon (Wallace et al., 2009). Indeed, work in the brain and vasculature uncovered the enzymes CAT and 3MST as a 3rd pathway for H_2S synthesis (Shibuya et al., 2009a; Shibuya et al., 2009b). This pathway functions independently of P5P and instead requires the presence of α-ketoglutarate. In this present thesis we examined the presence of this pathway in the colon and the contributions it makes to H_2S synthesis in health and disease.

We found that the healthy colon produced H_2S in the presence of α-ketoglutarate but the absence of P5P, and inhibitors of CAT suppressed this H_2S production. These data suggest that this 3rd pathway for H_2S production is present in the colon. We also found that H_2S production from the CAT-3MST pathway was markedly increased by day 3 after the induction of colitis in rats using DNBS. Importantly, we observed that in both the healthy and inflamed colon, CAT-3MST made greater contributions to colonic H_2S synthesis than CBS/ CSE, thus representing the major pathway for H_2S synthesis in the colon. Furthermore, increased production of H_2S via CAT-3MST occurred at the site of mucosal ulceration.

Using CSE-knockout mice we also found that CSE is the major contributor to P5P-dependent H_2S production in the colon. This finding is contrary to previous reports using inhibitors of CBS and CSE (Martin et al., 2010), but agrees with the notion that CSE is

expressed in peripheral tissue while CBS is mainly expressed in the central nervous system (Paul and Snyder, 2012). Furthermore, we also found that in the ulcerated mucosa, these P5P-dependent enzymes make important contributions to H_2S synthesis. Although not the main source of H_2S synthesis in the colon, it is possible that these pathways are inducible and make important contributions during times of damage and injury.

Lastly, we observed that the colon rapidly inactivated H_2S. In fact, colonic epithelial cells are very well adapted to the high luminal concentrations of H_2S in the colon and through mitochondrial activity can "detoxify" H_2S while also driving the production of adenosine triphosphate (ATP) (Goubern et al., 2007; Lagoutte et al., 2010). Sulfide quinone reductase (SQR), an enzyme capable of oxidizing H_2S into thiosulfate, thus inactivating H_2S, is highly expressed in the colonic mucosa (Linden et al., 2012). As we observed, this inactivation process is rapid and occurs within minutes and the majority of H_2S was broken down within 30 minutes. We also found that colonic tissue from rats with DNBS-induced colitis displayed an impaired ability to inactivate H_2S. The loss of the ability to inactivate H_2S appeared to occur at the site of ulceration. This may be of benefit to the site of damage as it allows for increased concentrations of H_2S, which has been shown to enhance ulcer healing, at the site of damage. This impaired ability to breakdown H_2S may also contribute to increased levels of H_2S measured during a bout of colitis in rats.

When taken together, the studies above further uncover important aspects of the colonic sources for H_2S synthesis. A better understanding of the pathways for the sources of H_2S production, its enzymatic pathways of production, and its metabolism will further uncover strategies for regulating this important mediator. The use of H_2S-donors to promote healing and the resolution of inflammation in animal models underscore the therapeutic potential of H_2S in the gastrointestinal tract. Moreover, inhibition of endogenous H_2S production retards both wound healing and the resolution of colitis. Thus, enhancement of endogenous H_2S synthesis may have clinical utility in treating gastrointestinal ulceration and inflammatory bowel disease (IBD).

5.2 Future Directions

Although enteric bacteria can be a significant source of H_2S, above we demonstrated that colonic bacteria do not contribute to what we measure of colonic H_2S synthesis. However, our results showed that bacteria in the feces were capable of producing H_2S from L-cysteine. Dietary L-cysteine that goes undigested by the small intestine and reaches the colon can be used as a substrate for H_2S production by colonic bacteria (Blanchier et al., 2010). Furthermore, we also found that during mucosal ulceration (using a hapten-model of colitis) the ability of the colon to breakdown H_2S is significantly impaired. Indeed, in a healthy state virtually all luminal H_2S that is not bound to feces is metabolized rapidly by enterocytes and colonocytes before reaching the subepithelial space (Lagoutte et al., 2010; Mimoun et al., 2012). However, during a

breach in the epithelial barrier as seen with hapten-induced colitis and reported in inflammatory bowel disease (IBD), it is possible that damage to this barrier could result in modulation of mucosal function and integrity by bacterial H_2S. This bacterial H_2S could have an effect on a variety of functions including epithelial secretion (Pouokam and Diener, 2011; Schicho et al., 2006), blood flow (Zhao et al., 2001), smooth muscle contractility (Hosoki et al., 1997), and mucosal defense (Blackler et al., 2012; Fiorucci et al., 2005; Wallace, 2010). Further studies will shed light on the effects of bacterial derived H_2S during breaches in the epithelial layer as seen in IBD.

Furthermore, the discovery of a 3rd pathway for H_2S synthesis in the colon provides another target for regulating local concentrations of H_2S. Finding novel ways to modulate the enzymatic pathways for H_2S production may have therapeutic benefit. For example, butyrate is produced by many species of bacteria in the gut (Blanchier et al., 2010; Sokol et al., 2008). There is also evidence that butyrate can stimulate the production of H_2S in colon cancer cells (Cao et al., 2010). Members of the endogenous microbiota such as *Bifidobacteria* and *Faecalibacterium prausnitzii* provide the epithelium with butyrate (Sokol et al., 2008). It may be possible that the beneficial effects of butyrate (Sokol et al., 2008), may be in part due to the ability to increase H_2S production. Other molecules may also display similar effects on H_2S production as butyrate, and could be investigated as new therapeutic interventions during colonic inflammation.

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APPENDIX

THIS THESIS IS A COMPILATION OF THE FOLLOWING MANUSCRIPTS:

Flannigan KL, McCoy KD, Wallace JL. Eukaryotic and prokaryotic contributions to colonic hydrogen sulfide synthesis. *Am J Physiol Gastrointest Liver Physiol* 301: G188-93, 2011.

Flannigan KL, Ferraz JGP, Wang R, Wallace JL. Alterations in hydrogen sulfide synthesis and degradation during experimental colitis. *In Preparation*, 2012.