HYDROGEN SULFIDE MODULATES HEPATIC OXYGEN AVAILABILITY AND MICROCIRCULATION DURING SEPSIS

by

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ABSTRACT

ERIC J. NORRIS. Hydrogen sulfide modulates hepatic oxygen availability and microcirculation during sepsis. (Under the direction of DR. MARK G. CLEMENS)

A mismatch between O_2 supply and demand leads to the development of tissue hypoxia which contributes to hepatic dysfunction and injury during sepsis. Previously, we reported that hepatic microcirculatory failure is a major contributor to hypoxia and hepatic dysfunction during sepsis. Recent evidence suggests that hepatic H_2S levels are increased during sepsis. Moreover, the inhibition of endogenous H_2S synthesis significantly improves survival in septic mice; however, the exact mechanism is not known. The present study was designed to investigate the effect of H_2S on hepatic O_2 availability and microcirculation during sepsis. We hypothesized that H_2S contributes to hepatic dysfunction during sepsis by reducing hepatic oxygen availability, via O_2 dependent oxidation of H_2S, and by potentiating microvascular dysfunction. We demonstrate that the liver is a central regulator of H_2S via mitochondrial oxidation during sepsis. Additionally, we show that the oxidation of H_2S lowers hepatic O_2 levels *in vivo*. In the second half of this study, we demonstrate that H_2S differentially affects the hepatic vascular response to phenylephrine and endothelin-1 which suggests that H_2S differentially affects presinusoidal and sinusoidal sites in the hepatic microcirculation. Using intravital microscopy, we show that portal infusion of H_2S is associated with sinusoidal constriction and that inhibition H_2S synthesis attenuates the sensitization of the sinusoids to the constrictor effect of endothelin-1 which improves perfusion. We conclude that the contribution of H_2S to hepatic tissue hypoxia and microcirculatory dysfunction is partially responsible for its detrimental effects during sepsis.

DEDICATION

To my beautiful and loving wife, Amy Roeder Norris.

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3-MST	3-mercaptopyruvate sulfurtransferase
ANOVA	analysis of variance
ATP	adenosine triphosphate
BAEC	bovine arterial endothelial cells
CAV-1	caveolin 1
CBS	cystathionine beta-synthase
cGMP	cyclic 3'-5' guanosine monophosphate
CLP	cecal ligation and puncture
CO	carbon monoxide
CSE	cystathionine λ lyase
DO_2	delivered O_2
eNOS	endothelial nitric oxide synthase
ET-1	endothelin-1
ET_A	endothelin A receptor
ET_B	endothelin B receptor
FITC	fluorescein isothiocyanate
GYY4137	H_2S donor, (p-methoxyphenyl)morpholino-phosphinodithioic acid
H_2DCF-DA	2',7'-dichlorodihydrofluorescein diacetate
H_2S	hydrogen sulfide
HO-1	heme oxygenase 1
HSC	hepatic stellate cells
IACUC	Institutional Animal Care and Use Committee

iNOS	inducible nitric oxide synthase
K_{ATP}	ATP sensitive-potassium channel
KC	kupffer cells
L-NAME	N^G-nitro-L-arginine methyl ester
LPS	lipopolysaccharide
MODS	multiple organ dysfunction syndrome
MOF	multiple organ failure
Na_2S	sodium sulfide
NaHS	sodium hydrosulfide
NAD^+	nicotinamide adenine dinucleotide (oxidized form)
NADH	nicotinamide adenine dinucleotide (reduced form)
NE	norepinephrine
NO	nitric oxide
NOS	nitric oxide synthase
O_2	oxygen molecule
PAG	dl-propargylglycine
PE	phenylephrine
PO_2	partial pressure of oxygen
SEC	sinusoidal endothelial cells
SEM	standard error of the mean
SIRS	systemic inflammatory response syndrome
$Ru(phen)_3^{2+}$	tris (1, 10-phenanthroline) ruthenium (II) chloride hydrate
VSMC	vascular smooth muscle cell

CHAPTER 1: INTRODUCTION

1.1 Overview

Hepatic failure is a significant complication during the late stages of sepsis; however, the first signs of hepatocellular dysfunction are observed early during disease progression and gradually progress to liver failure [19-20]. In the early stages of sepsis, cardiovascular function is characterized by a hyper-dynamic state, consisting of increased cardiac output and modest peripheral vasodilation [134]. During this period, overall delivery of O_2 (DO_2) to the liver is increased [27]. Despite the increase in DO_2, there is ample evidence demonstrating that hepatic injury is due in part to hypoxic stress [13, 28, 34, 41, 94]. While there is likely a contribution of mitochondrial dysfunction during sepsis [32], previous work from our lab and others demonstrate that disturbances in the hepatic microcirculation following inflammatory stress are a major contributor to hepatic dysfunction [12, 22, 63, 131]. During sepsis, the manifestations of hepatic microcirculatory dysfunction include heterogeneous tissue perfusion, focal hypoxia, and cell death. Eventually, an inability of the hepatic microcirculation to match O_2 supply with tissue demand can lead to hepatocellular injury, liver failure and the progression of sepsis.

As opposed to the macrocirculation which is responsible for transporting blood between organs, the microcirculation consists of smaller vessels (<100μm) that distribute blood within individual tissues. Arterioles are the primary resistance vessels in the

microcirculation, and due to their pre-capillary, location are responsible for modulating tissue perfusion [80]. Vascular smooth muscle cells (VSMCs) surround arterioles and act in functional syncytium in response to various stimuli to contract or relax, leading to a change in the vessel lumenal diameter. This change in vascular resistance can increase (relaxation) or decrease (contraction) perfusion through the capillaries. Since the resistance of a fluid to flow is inversely proportional to the fourth power of the lumenal diameter, small changes in vessel diameter can have profound effects on tissue perfusion.

Originally, it was thought that hepatic portal blood flow was solely regulated by total splanchnic drainage and pre-sinusoidal resistance vessels; however, it is now known that the hepatic sinusoid is also an important site of hepatic blood flow regulation. During sepsis, sinusoidal hyperconstriction results from the hypersensitization of the hepatic sinusoid to the vasoconstrictive effect of endothelin-1 (ET-1) [12, 94]. Sinusoidal hyperconstriction is associated with heterogeneous blood flow through the sinusoids resulting in a mismatch between O_2 supply and demand [13]. As a consequence, microcirculatory failure contributes to focal hypoxia and organ injury during sepsis.

The “gasotransmitters”, NO and carbon monoxide (CO), are important modulators of the hepatic microcirculation, particularly during sepsis [98, 108]. Recently, hydrogen sulfide (H_2S) has been added to the class of small, biologically active gases known as “gasotransmitters” [135]. It is now established that there are several biological functions common between H_2S, NO and CO, in particular their ability to cause VSMC relaxation and vasodilation [135]. Despite this common feature, the effect of H_2S on the hepatic oxygenation and microcirculation during sepsis remains unclear. As a vasodilator, one would predict that H_2S improves hepatic O_2 availability and microvascular function

following inflammatory stress. In support of this hypothesis, several studies demonstrate that H_2S provides a protective effect following ischemia/reperfusion injury [54, 73, 116, 127]. On the contrary, endogenous H_2S contributes to disease progression during sepsis [5, 144]. Since H_2S has been shown to alter cellular O_2 levels and modulate vascular resistance, the present study was designed to investigate the contribution of H_2S to hepatic oxygen availability and microvascular dysfunction during sepsis. Overall, we hypothesize that H_2S is metabolized in the liver at the expense of hepatic O_2 availability and contributes to hepatic sinusoidal sensitization to ET-1 and tissue hypoxia during sepsis. The findings of this study may provide evidence to partially explain why H_2S is deleterious during sepsis, despite being protective in several other pathophysiological conditions.

1.2 Hydrogen Sulfide

H_2S is a small, lipophilic gas commonly associated with the characteristic smell of rotten eggs. The first reports of a biological effect of H_2S predate those of the other "gasotransmitters". Nevertheless, only recently has H_2S garnered similar attention as a potential mediator in physiological processes [135]. Initial reports documented the lethality of exposure to elevated levels of the noxious gas, which was subsequently shown to result from potent inhibition of mitochondrial respiration. In a manner similar to cyanide poisoning, H_2S inhibits the activity of cytochrome *c* oxidase in the electron transport chain thereby preventing proper ATP synthesis [87].

For centuries, the toxicity of H_2S was the only known physiological effect in mammals. H_2S was simply considered metabolic waste produced by two pyridoxal 5' phosphate dependent enzymes, cystathionine β synthase (CBS) and cystathionine γ lyase

(CSE), during cysteine metabolism [18, 123]. The discovery that other gases act as important mediators in mammals led to the hypothesis that endogenous H_2S could be physiologically relevant. Support for this hypothesis was first provided by Abe and coworkers who demonstrated that low concentrations of the H_2S donor NaHS facilitated long term potentiation in rat hippocampus slices suggesting a function of H_2S in the nervous system [1]. A potential role in the cardiovascular system was demonstrated by Hosoki et al who reported H_2S caused relaxation of isolated aortic rings [48]. The observation that CSE $^{-/-}$ mice spontaneously develop hypertension confirmed that endogenous H_2S is a physiologically relevant mediator [140]. It is now well established that endogenous H_2S is an important mediator in the nervous, cardiovascular, gastrointestinal, pulmonary, and immune systems [136].

Even though there is a scientific consensus that H_2S is endogenously synthesized and a physiological important mediator, there is still considerable debate about several basic concepts in H_2S biology. First and foremost, the biologically active form of H_2S is not known. H_2S is a weak acid in aqueous solution with a pKa_1 between 6.6 and 7.1 and $pKa_2 > 12$ depending on experimental conditions [136]. Therefore, under physiological conditions, H_2S is in equilibrium with the deprotonated form, HS^-, with negligible amounts of S^{2-}. It is possible that H_2S, HS^-, or both exert biological functions. The different chemical properties of each form of sulfide may produce differential effects. For instance, the lipophilicity of H_2S allows for diffusion mediated movement across lipid membranes, whereas HS^- is not freely permeable, allowing for the possibility of compartmentalization of sulfide. Whether H_2S and HS^- exert differential effects has yet to

be determined. Therefore, as is currently used in H_2S biology, the terms H_2S and sulfide, are used to refer to both H_2S and HS^-, collectively, in this study [92].

The physiological concentration of H_2S *in vivo* remains unknown [92]. Because methods to measure H_2S levels are still developing, initial reports that identified the physiological circulating range to be between 10-300μM appear to be an overestimation [92]. Since even the lowest concentrations in this range would produce the characteristic rotten egg smell of H_2S in exhaled breath and blood, it is likely that circulating plasma H_2S levels are much lower or H_2S is bound in an undetectable form. In preliminary experiments, we were unable to detect any H_2S immediately after adding it to whole blood with the commonly used methylene blue H_2S assay. Interestingly, H_2S was easily detectable when added to plasma alone, suggesting an interaction between red blood cells and H_2S.

It is now thought that the level of H_2S *in vivo* is the result of local rates of H_2S production and disposal, which may be affected during disease states. The primary source of H_2S in the nervous system is CBS, while CSE is the main source of H_2S in the cardiovascular system and liver [1, 140]. Recently, a third enzyme, β mercaptopyruvate transferase (3MST), has been shown to produce a small amount of H_2S; however, the functional significance of this enzyme on total H_2S levels remains unclear [114]. CSE is constitutively expressed in the liver in several different cells including hepatocytes, hepatic stellate cells (HSC) and kupffer cells (KC) [33]. In addition, we have observed expression of CSE in isolated rat sinusoidal endothelial cells (data not shown). As the primary recipient of blood flow, the liver is subjected to a secondary source of H_2S from bacteria in the GI tract which synthesize H_2S as a metabolic byproduct producing H_2S

concentrations into the millimolar range [15]. This large, potentially toxic amount of sulfide is mostly sequestered and disposed in the feces; however, a small portion can escape the intestinal epithelial barrier and enter the portal circulation. Due to the combination of endogenous and exogenous sources of H_2S, it is likely that the liver is exposed to elevated levels of H_2S, particularly during sepsis. However, how the liver disposes of H_2S during sepsis remains unclear.

1.3 Hepatic hydrogen sulfide oxidation and sepsis

Treatment with bacterial endotoxin (LPS) leads to an increase in CSE expression in the macrophage like RAW 246.7 cell line suggesting that H_2S levels may increase during an inflammatory response [149]. In different *in vivo* models of sepsis, both cecal ligation and puncture and endotoxin treatment cause an increase in hepatic CSE expression which resulted in an increased hepatic capacity to synthesize H_2S [144]. In addition to elevated endogenous H_2S levels, damage to the intestinal cells has been shown to reduce their ability to detoxify H_2S which may permit more diffusion of H_2S into the portal circulation [129]. Moreover, in preliminary studies, we were able to detect measurable amounts of H_2S in the peritoneal fluid of rats following CLP supporting the hypothesis that there are elevated levels of bacterial derived H_2S during peritonitis.

Given that oxidative catabolism of toxins in the circulation is a main function of the liver, it is possible that the liver has the ability to dispose of H_2S to protect itself from toxic accumulation and prevent H_2S entry into the systemic circulation. Recently, Lagoutte et al reported that colonic epithelial cells metabolize H_2S via mitochondrial oxidation which protects them from toxic levels of sulfide derived from the gut bacteria [68]. Early studies on sulfide detoxification show that oxidation of H_2S occurs in a

recirculating isolated, perfused liver system with a concomitant increase in hepatic O_2 consumption [8]. Accordingly, several studies have demonstrated an inverse relationship between O_2 and H_2S levels in various experimental settings [91, 93, 138]. This led to the hypothesis that H_2S acts as a cellular oxygen sensor. When O_2 levels fall, H_2S oxidation is inhibited allowing for the accumulation of H_2S. At sufficient levels, H_2S would exert a biological function to raise O_2 levels. One possibility would be vasodilation of resistance vessels leading to an increase in tissue perfusion which would raise cellular O_2 levels. Once O_2 levels reach adequate levels, H_2S oxidation can resume and H_2S levels would fall [91]. In this scenario, the balance struck between O_2 and H_2S ensures adequate tissue perfusion. However, this scenario may be potentially detrimental in the septic liver.

During sepsis, impaired O_2 delivery and mitochondrial dysfunction leads to inefficient ATP production [13, 23]. Any physiological process that consumes hepatic O_2 may contribute to hepatic hypoxic stress. This raises the possibility that hepatic oxidation of H_2S during sepsis may exacerbate the hepatic tissue hypoxia. On the contrary, if the capacity to metabolize H_2S is inhibited during sepsis, then H_2S may accumulate to toxic levels and contribute to hepatic mitochondrial dysfunction via inhibition of cytochrome c oxidase. Therefore, the first part of this study was designed to test the capacity of the liver to metabolize H_2S and to determine if H_2S oxidation remains a priority during sepsis.

1.4 H_2S and hepatic microcirculation during sepsis

It is known that failure of the microcirculation is a major contributor to the progression of sepsis. Previous work from our lab and others focused on the important contribution of NO in both systemic and hepatic microcirculatory dysfunction [47, 95-97,

124]. Under normal conditions, resistance vessels respond to local and humoral vasoregulators to modulate tissue perfusion. During sepsis, the resistance vessels become hyporesponsive to the vasoconstrictive effect of catecholamines due to the excessive production of vasodilators. In an isolated, perfused liver, treatment with endotoxin attenuates the increase in intrahepatic resistance in response to phenylephrine (PE) [97]. This effect was reduced when livers were treated with the nitric oxide synthase (NOS) inhibitor L-N^G-nitroargininie methyl ester (L-NAME), indicating the important role of NO in the hyporesponsiveness to catecholamine signaling. The dysregulation of blood flow distribution leads to heterogeneous organ perfusion which contributes to organ failure during sepsis [52]. While clinical trials targeting NO production have demonstrated a decrease in systemic hypotension, they have failed to significantly improve septic patient outcomes [77].

The regulation of sinusoidal perfusion is more complex than capillary perfusion in other vascular beds. At presinusoidal sites, the resistance of portal terminal venules and hepatic arterioles is modulated by the contractility of VSMCs similar to other vascular beds. In the liver, the hepatic sinusoid participates in the regulation of tissue perfusion as well [11]. Individual sinusoids lack VSMCs; rather they are surrounded by hepatic stellate cells (HSCs) [79]. These specialized pericytes can respond to local vasoactive agents and modulate sinusoidal resistance [25, 62]. HSC contraction reduces sinusoidal diameter and decreases flow through individual sinusoids [146]. As a consequence, sinusoidal constriction is characterized by the juxtaposition of constricted and dilated sinusoids and heterogeneous sinusoidal flow [57].

ET-1 is a potent 21 amino acid vasoactive peptide which is synthesized by SECs, HSCs, and KCs in the liver [106, 139]. The physiological effects of ET-1 are dependent on two receptor subtypes, ET_A and ET_B receptors, which are heterogeneously distributed in the liver [42]. HSCs primarily express ET_A receptors, while SECs express both ET_A and ET_B receptors [49]. ET-1/ET_A interaction primarily mediates vasoconstriction, while ET-1/ET_B interaction produces both vasoconstriction and vasodilation [10, 46]. Importantly, ET-1 levels are elevated following inflammatory stress, including sepsis, and are correlated with disease severity [3, 119]. Extensive work from our lab has demonstrated the importance of the antagonistic relationship between ET-1 and NO in modulating the hepatic microcirculation [98].

Zhang et al was the first to demonstrate that sinusoidal constriction in response to ET-1 co-localizes with HSCs *in vivo* [146]. ET-1 and PE both increased hepatic resistance and decreased portal inflow; however, only ET-1 had an effect on sinusoidal diameter. Moreover, inflammatory stress primes the liver to the vasoconstrictive effect of ET-1 [94, 107]. The potentiated response of the hepatic sinusoid to ET-1 leads to microcirculatory dysfunction and impaired O_2 delivery [12]. Additionally, hypersensitization to ET-1 during sepsis is associated with an increase in the heterogeneity of sinusoidal perfusion resulting in focal hypoxia and hepatic injury [13].

Mechanistic studies have demonstrated the importance of eNOS-derived NO from SECs in maintaining sinusoidal tone [112]. Under normal conditions, the vasoconstrictive effect of ET-1 in HSCs is mitigated by NO produced in SECs [98]. The ET-1/ET_B interaction increases the activity of eNOS in isolated, sinusoidal endothelial cells and maintains tissue oxygenation *in vivo* [67, 98]. During sepsis, the balance between the

vasoregulatory effects of ET-1 is skewed resulting in hypersensitization of the hepatic sinusoid to the vasoconstrictive action of ET-1. This imbalance is largely due to an impairment of ET-1 stimulated eNOS activation [67]. While there is an increase in the ratio of ET_B receptors to ET_A receptors, an increase in the structural protein caveolin-1 inhibits eNOS activity and NO bioavailability [113, 119]. The importance of ET_B receptor activation during sepsis is supported by studies which demonstrate a beneficial effect in the hepatic microcirculation of an ET_B agonist in LPS-treated animals [98]. Moreover, administration of the nitric oxide donor, sodium nitroprusside, improves microcirculatory dysfunction following injury suggesting the importance of NO in maintaining sinusoidal perfusion [41].

In livers isolated from normal and cirrhotic rats, Fiorucci et al demonstrated that H_2S attenuates the vasoconstrictive effect of norepinephrine (NE) via its actions as a vasodilator. Since portal hypertension is a significant complication during cirrhosis and fibrosis, the decrease in intrahepatic resistance would be beneficial [33]. Unlike cirrhosis, portal hypertension is not a significant complication during sepsis. Rather, impaired O_2 delivery due to sinusoidal hyperconstriction is the major complication in liver injury during sepsis. As a vasodilator, one would predict that H_2S would mitigate sinusoidal constriction. However, sinusoidal constriction is observed in the hepatic microcirculation despite elevated H_2S levels. Therefore, it is possible that H_2S does not act as a vasodilator in the hepatic sinusoid.

H_2S-mediated vasodilation is mainly the result of activation of K_{ATP} channels expressed in VSMCs [148]. K_{ATP} channel activation produces hyperpolarization and relaxation [103]. H_2S would only be beneficial in the liver during sepsis if it acts as a

vasodilator on the sinusoids; however, the hepatic sinusoids lack VSMCs. Therefore, H_2S may exert a different effect in the sinusoid. Moreover, there are several reports that H_2S can produce vasoconstriction [74, 76]. If H_2S exerts a different effect on presinusoidal and sinusoidal sites of regulation, then it would differentially affect the vascular responses to site specific vasoactive molecules in an isolated perfused liver. Therefore, the second part of this study was designed to investigate the effect of H_2S on PE and ET-1 infusion during endotoxemia to determine if there is a differential effect of H_2S at presinusoidal and sinusoidal sites of hepatic blood flow regulation.

To determine if H_2S differentially regulated presinusoidal and sinusoidal sites, we used an isolated, perfused liver system. The advantage of this experimental technique is that it allows for accurate monitoring of changes in intrahepatic resistance during constant flow perfusion. PE acts on VSMC cells in the portal terminal venules, whereas ET-1 exerts its effect at sinusoidal and presinusoidal sites. Since endotoxemia produces hypersensitization of the sinusoids to ET-1, we can identify the location of an H_2S effect by comparing the effects of PE and ET-1 infusion. However, since changes in inflow pressure correlate to total intrahepatic resistance, we could not directly assess the sinusoids. Moreover, heterogeneous sinusoidal flow is a critical component in the development of focal hypoxia and liver injury during sepsis. *In vivo* microscopy is a more laborious technique that allows for the direct visualization of the hepatic microvasculature and determination of flow through the sinusoids. Therefore, the last part of this study was performed to test the contribution of endogenous H_2S to hypersensitization of the hepatic sinusoid to ET-1 and hepatic tissue perfusion heterogeneity during endotoxemia.

CHAPTER 2: THE LIVER AS A CENTRAL REGULATOR OF HYDROGEN SULFIDE

2.1 Abstract

The liver is likely exposed to high levels of H_2S from endogenous hepatic synthesis and exogenous sources from the gastrointestinal tract. Little is known about the consequence of H_2S exposure on the liver or hepatic regulation of H_2S levels. We hypothesized that the liver has a high capacity to metabolize H_2S and that H_2S oxidation is decreased during sepsis; a condition in which hepatic O_2 is limited and H_2S synthesis is increased. Using a non-recirculating isolated and perfused liver system, we demonstrated rapid hepatic H_2S metabolism up to an infusion concentration of 200μM H_2S. H_2S metabolism was associated with an increase in O_2 consumption from a baseline 96.7 ± 7.6 μmoles O_2/min/kg to 109 ± 7.4 μmoles O_2/min/kg at an infusion concentration of 150 μM H_2S ($P<0.001$). Removal of O_2 from the perfusate decreased H_2S clearance from a maximal 97% to only 23%. Livers isolated from rats subjected to cecal ligation and puncture (CLP) did not differ significantly from control livers in their capacity to metabolize H_2S suggesting that H_2S oxidation remains a priority during sepsis. To test whether H_2S induces O_2 consumption *in vivo*, intravital microscopy was utilized to monitor the oxygen content in the hepatic microenvironment. Infusion of H_2S increased the NADH/NAD^+ ratio (645 grey scale unit increase, $P=0.035$) and decreased hepatic O_2 availability visualized with $Ru(Phen)_3^{2+}$ (439 grey scale unit increase, $P=0.040$). We conclude that the liver has a high hepatic capacity for H_2S metabolism. Moreover, H_2S

oxidation consumes available oxygen and may exacerbate the tissue hypoxia associated with sepsis.

2.2 Introduction

For centuries, hydrogen sulfide (H_2S) has been known almost exclusively as a toxic gas associated with the characteristic smell of rotten eggs. However, recent evidence now demonstrates that H_2S is an endogenously produced biologically active gaseous molecule [135]. Numerous studies have identified H_2S as an important mediator in several biological systems including neurological, cardiovascular, and gastrointestinal systems [15, 29, 122, 140]. The role of H_2S in inflammation remains poorly defined [84, 116, 142-145]. H_2S is synthesized during cysteine metabolism via two pyridoxal - 5'-phosphate dependent enzymes; cystathionine β-synthase (CBS) primarily in the brain and cystathionine γ-lyase (CSE) primarily in the vasculature and liver [18, 123]. Additionally, the synthesis of H_2S by the microflora of the gastrointestinal system provides an exogenous source of H_2S that may enter the portal circulation [15]. The liver is uniquely positioned to be exposed to high levels of H_2S; however, how the liver responds to elevated hydrogen sulfide levels is unclear. Several studies have observed circulating hydrogen sulfide levels between < 1 and 300 μM. Anecdotal evidence suggests that it is unlikely that H_2S circulates in the blood within this range as neither blood nor expired air smell of the characteristic rotten egg odor that would be expected from these levels of H_2S [92]. Therefore, we propose that the liver, as a consequence of its location, is a key regulator of H_2S levels by maintaining a high capacity for H_2S clearance from the circulation

Despite extensive research, sepsis remains a significant clinical problem today accounting for 1.3 percent of all hospitalizations [78]. Sepsis is associated with an inflammatory cascade resulting in hepatic dysfunction [19]. Failure of the hepatic microcirculation results in inadequate perfusion resulting in heterogeneous oxygen distribution and tissue hypoxia [12, 121]. Several studies have demonstrated cellular metabolism of H_2S via oxidation in the mitochondria [8, 68]. The oxidation of H_2S during sepsis could have detrimental unintended consequences during sepsis as it would compete for already limited oxygen resources. Moreover, in the absence of mitochondrial oxidation, H_2S levels could accumulate to potentially toxic levels in the liver and enter the general circulation.

Currently there is very little of information in the literature regarding the effect of H_2S on the hepatic microenvironment, particularly during pathological conditions, such as sepsis, where oxygen availability is limited and H_2S synthesis is increased [144]. The role of H_2S during sepsis is still unclear and a better understanding is vital for the potential development of targeted therapeutic intervention [45] Therefore, the aim of the present study was to assess the capacity of the liver to metabolize H_2S with particular emphasis on the relationship between H_2S oxidation and hepatic oxygen availability. The study was also designed to investigate whether the capacity for H_2S oxidation would be greatly decreased during sepsis due to hepatocellular dysfunction and limited oxygen availability. The findings of this study highlight the delicate balance between H_2S clearance and hepatic oxygen availability.

2.3 Materials and Methods

Animals**:** Male Sprague-Dawley rats (Charles River Laboratories, Fayetteville, NC) were housed in a temperature-controlled setting under 12-hour light/dark cycles. Rats were maintained on standard rat chow or fasted overnight with free access to water depending on experimental conditions. All animal manipulation was in strict adherence with National Institutes of Health guidelines and experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Charlotte.

Isolated and perfused liver preparation**:** Preparation of the non-recirculating liver perfusion system followed that of Sugano et al with slight modifications [125]. Briefly, rats were anesthetized under light isoflurane. A laparotomy was performed to expose the abdominal cavity. Heparin (1000 units/kg body) was injected into the inferior vena cava. The portal vein was cannulated and perfused with warm, oxygenated Kreb's Henseleit buffer [NaCl 118.95mM, $MgSO_4$ 1.2mM, KH_2PO_4 1.2 mM, KCl 4.65 mM, $NaHCO_3$ 25 mM, EDTA 0.1 mM, $CaCl_2$ 2.5mM, $C_3H_6O_3$ (lactic acid) 5mM, $C_3H_3O_3Na$ (pyruvic acid) 1 mM, $C_6H_{12}O_6$ (glucose) 5mM, pH= 7.4] [2]. The flow rate was held constant and calculated as 16ml/minute/100 grams body weight. The thorax was opened and a cannula was inserted into the inferior vena cava to allow for collection and monitoring of the effluent. Lastly, the vena cava was ligated above the renal vein to close the system and ensure perfusate through the effluent cannulation. The liver was perfused *in situ* with warm, oxygenated Kreb's buffer for a period of 20 minutes to allow for stabilization. Oxygen consumption and temperature were monitored during the stabilization period to ensure proper preparation. Only livers that demonstrated stable O_2 consumption during

the stabilization period were considered satisfactory preparations and used in future experiments.

Na_2S preparation**:** The H_2S donor Na_2S (Sigma-Aldrich, St. Louis, MO) was used in all experiments. Na_2S completely dissociates in water to the weak conjugate base S^{2-} which combines with available H^+ ions to establish an equilibrium between $H_2S \leftrightarrow HS^- + H^+$ at physiological pH. It remains unclear which form of H_2S is biologically active. For clarity, the term H_2S will be used to represent all sulfide added to the system from Na_2S. Na_2S was dissolved in the appropriate buffer immediately before use as H_2S is a volatile gas with a short half life. All standard curves were made at the time of experimental collection and all samples were stored in the exact conditions.

H_2S clearance**:** Following the stabilization period, H_2S was infused into the influent at a rate of 1% of the of total flow rate resulting in final H_2S concentrations between 0-500 μM in 50 μM increments. Preliminary experiments demonstrated a rapid equilibration H_2S concentration in the perfusate. Thus, infusion of H_2S lasted for 2.5 minutes. A 2.5 minute recovery period was used to give ample time to return to baseline. Inflow and outflow perfusates were sampled in duplicate 90 seconds after H_2S infusion began. Samples were immediately transferred into 1.5 ml eppendorf tubes containing 150 μl of 1% zinc acetate to trap the H_2S in solution. All samples were frozen and stored at -80 Celsius overnight before the H_2S assay.

For experiments requiring different levels of buffer oxygenation, Kreb's buffer was bubbled with a combination of gases (N_2, O_2, CO_2) to achieve the desired level prior to the experiment. Oxygen levels were monitored for a period of 20 minutes to ensure the combination of gases resulted in a stable level of oxygenation.

Oxygen Monitoring**:** The oxygen content of the perfusate was monitored using an oxygen sensitive cathode connected to an oxygen monitor (YSI Life Sciences, Yellow Springs, Ohio). All measurements were recorded continuously using Biopac systems MP 100 transducer and software (Goleta, CA). Oxygen sensors were calibrated daily with distilled H_2O bubbled with ambient air. Inflow perfusate oxygen concentration was measured immediately prior to and after the experiment. Only experiments where inflow oxygen content remained constant throughout the experiment were used.

Isolated and Perfused Heart Preparation**:** Surgical preparation was done as described in Clemens et al with slight modifications [24]. Briefly, the excised heart was quickly transferred to a petri dish containing ice cold Kreb's buffer. The aorta was quickly cannulated and secured into position with suture material. Warm, oxygenated Kreb's buffer was perfused through the heart at a flow rate of 10 ml/min/g tissue. The pulmonary artery was cannulated to allow collection of outflow sample. The entire organ preparation was suspended inside a temperature-controlled perfusion chamber. A 20 minute period of stabilization occurred prior to the start of the experiment. Infusion with H_2S occurred in exactly the same manner as with the isolated liver perfusion preparations.

Phenylephrine Treatment**:** Livers isolated from 18 hour fasted rats were prepared as described. Lactate, pyruvate, and glucose were removed from the Kreb's buffer for assessment of baseline gluconeogenesis. After 20 minute of stabilization, the perfusion buffer was switched to buffer containing the gluconeogenic substrates lactate (5mM) and pyruvate (1mM) with or without 100 μM H_2S. Isolated livers were then stimulated with the alpha adrenergic agonist L-phenylephrine (PE) (Sigma-Aldrich, St. Louis, MO). A

maximum vasoconstrictor response was observed at a PE infusion concentration of 5 μM. This concentration was chosen for comparison of gluconeogenesis and vasoactivity between control and the experimental H_2S group.

Gluconeogenesis assessment**:** Glucose production from isolated perfused livers was measured using effluent samples collected and stored at -80^{o}C. The concentration of glucose in samples was determined using the colorimetric glucose oxidase and peroxidase method (PGO enzymes and dianisidine, Sigma-Aldrich, St. Louis, MO). Samples were incubated with the enzyme solutions for 30 minutes at 37^{o}C at and then read at 450nm on a Beckman Spectrophotometer. Glucose concentrations were calculated from a standard curve of known values.

H_2S assay**:** 75 μl samples were immediately placed in 150 μl of 1% zinc acetate to trap H_2S in solution. To this solution, 133 μl of 20 mM N,N- dimethyl-p-phenylenediamine sulfate (dissolved in 7.2 M HCl) and 133μl 30 mM $FeCl_3$ (dissolved in 1.2 M HCl) was added and then the solution was vigorously vortexed. After 20 minute incubation at room temperature, addition of 300 μl of 10% trichloroacetic acid was followed by centrifugation (10,000g, 5 min 4° Celsius) to precipitate proteins. Absorbance was measured at 670 nm. H_2S levels were calculated against a standard curve. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Polymicrobial sepsis model**:** Cecal Ligation and puncture was used to induce polymicrobial sepsis. Fasted rats were anaesthetized under light isoflurane. A small midline incision was made to allow for exteriorization of the cecum. The cecum was carefully ligated to ensure unobstructed movement of digested food through the GI tract. Double puncture of the cecum was performed using a 22 gauge needle. The cecum was

gently squeezed to extrude a small amount of feces into the peritoneal cavity. The cecum was carefully placed back into the peritoneal cavity and the incision closed. Animals received 15ml of saline subcutaneously after the surgical procedure to prevent dehydration. Implantable electronic temperature transponders (Bio Medic Data Systems, Seaford, Delaware) were placed subcutaneously to allow periodic monitoring of the animal. Sham animals received all surgical manipulations with the exception of ligation and puncture of the cecum. 24 hours after surgery was performed the liver was isolated and H_2S clearance was assessed as previously described.

Intravital Microscopy**:** Fasted rats were anaesthetized using sodium pentabarbital (50mg/kg) (Lundbeck Inc., Deerfield, IL). The abdominal cavity was opened to expose the liver and portal vein. The splenic vein was cannulated to allow for infusion of H_2S with minimal disruption of portal flow. The external jugular vein was cannulated for infusion of the oxygen sensor Tris (1, 10-phenanthroline) ruthenium (II) chloride hydrate ($Ru(phen)_3^{2+}$ (Sigma-Aldrich, St. Louis) and for administration of booster doses of pentabarbital as needed. The left carotid artery was cannulated to monitor mean arterial blood pressure (Digi-Med high-pressure analyzer; Micro-Med, Louisville, KY). Heart rate and blood O_2 saturation were continuously monitored using a MouseOx pulse oximeter (Starr Life Sciences, Pittsburgh, PA) to ensure stable preparation. Prepared rats were placed on an Olympus Ix70 inverted microscope (Olympus America, Melville, NY). Saline was first infused at a rate of 50 µl/minute for 10 minutes to determine the effect of infusion on hepatic oxygen distribution. Short acting alpha adrenergic agonist phenylephrine (0.15µmoles/min/kg) was infused for ten minutes to ensure proper experimental and camera setup. Only rats that responded to PE stimulation with a clear

decrease in hepatic O_2 levels were considered satisfactory preparations. Saline was infused for 20 minutes to allow hepatic oxygen distribution and systemic indicators (mean arterial pressure, heart rate, and blood O_2 saturation) to return to baseline. H_2S was infused into the portal circulation at a rate of 2 μmoles/min/kg for 10 minutes to achieve an estimated plasma concentration of 200μM. A 10 minute recovery period with saline infusion immediately followed H_2S infusion.

Assessment of oxygen distribution *in vivo* was performed as previously described [99]. The liver was exteriorized onto a glass coverslip to allow for epi-illumination of the surface of the left lobe. NADH fluorescence (excitation 366nm, emission 450 nm and $(Ru(phen)_3^{2+})$(excitation 480 nm, emission 625 nm) were captured using a Cooke Sensicam digital CCD camera (The Cooke Corporation, Romulus, MI) and PTI Imagemaster software. All gain, black level, and contrast enhancements were exactly the same throughout each experiment.

Statistical Analysis**:** The data are presented as means ± standard error (SE). Statistical significance was determined using ANOVA or linear regression analysis. The Student-Newman-Keuls *post hoc* test was used when ANOVA analysis detected significance. Independent and repeated measures analysis was used where appropriate. Data not passing the normality test were log transformed to achieve normality. Statistical significance was $P < 0.05$.

2.4 Results

The liver has a high capacity for clearance of H_2S**:** To determine if the liver is capable of clearing H_2S from the circulation, a non-recirculating isolated and perfused liver system was utilized. Livers isolated from rats were perfused with Kreb's buffer and

subjected to increasing infusion concentrations of the H_2S donor compound Na_2S to achieve final perfusate H_2S concentrations of 50-500μM. The inflow and outflow perfusates were collected and sampled for H_2S levels. The H_2S clearance ratio (outflow perfusate [H_2S]/inflow perfusate [H_2S]) was used as a measure of the hepatic ability to clear H_2S at each concentration. Perfusion with Kreb's buffer alone resulted in undetectable levels of H_2S in the outflow perfusate indicating no net H_2S synthesis occurring in the liver (data not shown). Infusion of Na_2S resulting in final concentrations of 50, 100, 150, and 200 resulted in a near complete ability to clear H_2S as indicated by 97%, 95%, 97%, and 91% clearances respectively (Figure 1). Fifty percent H_2S clearance was observed at an infusion concentration of 303 ± 28 μM. At H_2S perfusate concentration greater than 400 μM, an almost complete inhibition of H_2S clearance was observed (Figure 2). To determine if H_2S clearance is a general cellular capability or potentially site specific, we employed a non-recirculating isolated and perfused heart system to compare to hepatic H_2S clearance. Na_2S was infused to achieve final perfusate concentrations in the physiological relevant range (0-200μM). In isolated hearts, a linear correlation exists between the concentration of H_2S in the inflow perfusate and outflow perfusate (Figure 3, R^2=0.979, P<0.001). A linear regression line slope of 1 is expected if no H_2S is removed from the perfusate. For isolated hearts, the slope of the regression line was 0.751 indicating very little removal of H_2S from the perfusate by the heart. Linear regression analysis from isolated livers had a slope of 0.266 indicating nearly complete removal of H_2S from the perfusate. Importantly, this slope was not statistically different from zero over the physiological range (R^2=0.266, P=0.071).

Effect of oxygen availability on hydrogen sulfide metabolism**:** To investigate whether H_2S is being metabolized by the liver or simply bound and sequestered, inflow and outflow perfusate oxygen tension was monitored in isolated and perfused livers. Inflow perfusate was oxygenated for 20 minutes prior to experiment resulting in an inflow oxygen tension of 593.50 ± 28.47 mmHg. Isolated livers consumed 96.7 ± 7.6 μmoles O_2 / min / kg body weight when perfused with Kreb's buffer alone. Infusion with Na_2S resulting in final H_2S concentrations of 50, 100, 150 and 200 μM significantly increased O_2 consumption to 104 ± 7.6 ($P<0.001$), 107 ± 7.7 ($P<0.001$), 109 ± 7.4 ($P<0.001$) and 105 ± 7.4 ($P<0.001$) μmoles O_2 / min / kg body weight respectively (Figure 4). Representative tracings of oxygen consumption at infusion concentrations of 150, 250, and 350 μM demonstrate the shift from increased O_2 consumption to the toxic inhibitory effect of H_2S on oxidative metabolism. Infusion with 250 μM H_2S-induced a biphasic response with an initial increase in O_2 consumption followed by a dramatic decrease in O_2 consumption, due to the accumulation of H_2S during infusion (Figure 5). At pathological H_2S concentrations greater than 250 μM, an increasing inhibition of oxygen consumption was observed with 0_2 consumption being 74% of maximal consumption at 300μM and 46% of maximal consumption at 500μM. The requirement of O_2 for H_2S metabolism was tested by altering the oxygen content of the perfusate. Kreb's buffer was oxygenated to establish perfusate oxygen partial pressures of 593.50 ± 28.47, 245.72 ± 38.42, and 21.0 ± 2.52 mmHg O_2. Isolated livers perfused with the high oxygen content perfusate metabolized 50% of the H_2S at an inflow concentration of 303 ± 29.5 μM. Perfusion with buffer containing approximately 300 mmHg O_2 resulted in a significant leftward shift in the 50% metabolism point to 221.7 ± 5.84 μM, $p<.05$ (Figure

6). Decreasing the oxygen content to 150 mmHg further lowered the 50% metabolism point to 170.0 ± 23.6 μM. An essential role of oxygen availability in H_2S metabolism was observed as Kreb's buffer bubbled with 95% N_2 / 5% CO_2 rendered the liver incapable of metabolizing 50% of the H_2S at any concentration. At the lowest H_2S infusion concentration (50μM) only 23% of the infused hydrogen was metabolized by the liver.

Hydrogen sulfide affects the vascular and metabolic response to phenylephrine**:** Given the rapid metabolism of H_2S by the liver, we tested whether H_2S was able to exert physiological effects on the liver during perfusion. Livers isolated from 18 hour fasted rats were treated with the alpha adrenergic agonist phenylephrine (5μM) and gluconeogenic substrates lactate (5mM) and pyruvate (1mM). After a 20 minute stabilization period with incomplete Kreb's buffer, isolated livers were perfused with complete Kreb's (addition of lactate and pyruvate) buffer containing phenylephrine. Immediately before addition of complete Kreb's buffer, baseline levels for portal pressure (3.96 ± 0.19 mmHg), oxygen consumption (1.824 ± 0.165 μmoles O_2 / min / g liver wet weight), and glucose production (0.254 ± 0.11 μmoles glucose / min / g liver wet weight) were recorded. Addition of complete Kreb's without phenylephrine did not affect portal pressure (data not shown). The addition of phenylephrine increased portal pressure to 9.3 ± 0.6 mmHg (Figure 7). In the presence of 100 μM H_2S, the phenylephrine-induced increase was significantly attenuated (5.7 ± 0.2 mmHg, $P<0.001$). The effect of H_2S on gluconeogenesis was determined by measuring the glucose levels in the outflow perfusate. Glucose production increased from baseline (0.254 μmoles / min / g liver wet weight) in control to 0.957 ± 0.11 ($P<0.001$) μmoles / min / g liver wet weight ($P<0.001$) following stimulation with phenylephrine and gluconeogenic substrates (Figure 8).

Addition of H_2S did not significantly affect the increase in glucose output (1.314 ± 0.26, P=.084). Since vascular changes and gluconeogenesis have an effect on oxygen levels, the partial pressure of oxygen was measured to determine effects on oxygen consumption. Addition of phenylephrine and gluconeogenic substrates resulted in a 28% increase in oxygen consumption over baseline (Figure 9, $P<0.05$). A significantly greater increase in oxygen consumption was observed when H_2S was combined with phenylephrine and substrates (80% over baseline, P=0.005).

Hydrogen Sulfide metabolism remains a priority during sepsis**:** Cecal ligation and puncture (CLP) was performed to investigate whether hydrogen sulfide metabolism is altered during polymicrobial sepsis. Rats were subjected laparotomy followed by double puncture CLP with a 22 gauge needle. Sham control rats underwent laparotomy without CLP. After 24 hours, isolated livers were perfused with increasing concentrations of H_2S (50-500 μM). Inflow and outflow perfusates were monitored for H_2S levels and oxygen tension. Isolated livers from sham and CLP treated rats showed no significant difference in hydrogen sulfide clearance or oxygen consumption. Sham and CLP treated livers had 50% clearance concentrations of 198 ± 6.7 and 196 ± 35 μM respectively (Figure 10). Sham treated livers reached a maximal H_2S clearance rate of 17.2 ± 1.6 μmoles/ min/ kg body weight at an infusion concentration of 132 μM (Figure 11). CLP treated livers had a maximal H_2S clearance rate of 16.9 ± 1.9 17.2 ± 1.6 μmoles/ min/ kg body weight at an infusion concentration of 127 μM. Similarly, the oxygen consumption in sham and CLP livers did not differ with an increase O_2 consumption at low H_2S infusion (<150 μM) concentrations and a decrease in oxygen consumption at high H_2S levels (>200 μM) (Figure 12).

H_2S oxidation induces hepatic tissue hypoxia *in vivo:* Experiments using a non-recirculating isolated and perfused liver system are standard practice for monitoring of hepatic function, however they are limited. Kreb's buffer is a much simpler perfusate than blood. Furthermore, non-physiological flow rates are required to provide ample oxygenation to the tissue leading to increased shear stress. Therefore, we investigated the effect of H_2S infusion on hepatic oxygen distribution *in vivo* using intravital microscopy. The splenic vein was cannulated for infusion of H_2S directly into the portal circulation for minimal disruption of portal flow. The surface of the liver was illuminated in order to assess the fluorescence of two separate markers of oxygen availability. An increase in NADH fluorescence (excitation 366nm, emission 450 nm) represents in an increase in the $NADH/NAD^+$ ratio. Since oxygen is the final electron acceptor in the electron transport chain, a loss of O_2 would result in an increase in the $NADH/NAD^+$ ratio. As H_2S is an inhibitor of the electron transport chain, it is possible that it could raise $NADH/NAD^+$ independently of available oxygen. Therefore, the oxygen sensor molecule $Ru(Phen)_3^{2+}$ (excitation 480 nm, emission 625 nm) was used as a direct measurement of oxygen distribution. Infusion of saline alone resulted in no change in the mean micrograph grey scale level in either NADH or $Ru(Phen)_3^{2+}$ fluorescence indicating stable oxygen distribution during infusion . When H_2S was infused at a predicted final plasma concentration of 200 μM, a significant increase in the mean micrograph grey scale level was observed in NADH fluorescence (645 grey scale unites, P=0.035, Figure 13) and $Ru(Phen)_3^{2+}$ (439 grey scale unites, P=0.040, Figure 14) following 10 minutes of H_2S infusion, indicating that H_2S oxidation reduces the PO_2 in the hepatic microenvironment.

2.5 Discussion

Originally known for its cytotoxicty and pungent odor, H_2S is now recognized, along with nitric oxide and carbon monoxide, as the third major gasotransmitter with important physiological roles in neurological [29], cardiovascular [3], and gastrointestinal function[15]. H_2S is synthesized endogenously during cysteine metabolism via two pyridoxal - 5'-phosphate dependent enzymes: cystathionine β-synthase (CBS) primarily in the brain and cystathionine γ-lyase (CSE) primarily in the vasculature and liver, [18, 123] resulting in physiological levels of H_2S reported to be between < 1 and 300 μM [92]. Exogenous H_2S is synthesized during the normal cellular metabolism of microflora in the gastrointestinal system and may enter the portal circulation [15]. As the major recipient of gastrointestinal blood flow, the liver is uniquely positioned to be subject to high levels of H_2S from a combination of endogenous and exogenous sources. Furthermore, H_2S synthesis is increased during pathological conditions including sepsis [144]. Given that a primary function of the liver is the oxidative metabolism of toxins in the circulation, we tested the hypothesis that the liver is a critical site of metabolism of H_2S from the circulation in an oxygen dependent manner. Additionally, we investigated whether hydrogen sulfide metabolism is altered during polymicrobial sepsis; a pathology in which H_2S levels are increased and limited oxygen availability is a contributing factor[121].

The hypothesis that the liver is capable of clearing H_2S via oxidation from the circulation is not new [8]. However, those experiments were conducted using a blood recirculating isolated and perfused liver system over a period of 15 minutes making it difficult to discount any possible contribution sequestration by red blood cells on the rate

of H_2S clearance. Since circulation time in the rat is about 1 minute, we sought to measure the capacity of the liver to metabolize hydrogen sulfide dissolved in oxygenated Kreb's buffer during a single pass through an isolated liver. Our findings demonstrate that the liver clears virtually all hydrogen sulfide at perfusate concentrations below 200μM (Figure 1). This ability appears to be constant as the liver was able to clear 100μM H_2S from the perfusate for over an hour in preliminary experiments (data not shown); a finding in agreement with the original H_2S clearance study.

Controversy exists over the physiologically relevant concentrations of circulating H_2S in blood with values ranging two orders of magnitude [92, 127]. The findings of the present study provide strong evidence against the circulation of free sulfide in the general circulation. As the recipient of 20% of cardiac output, the liver rapidly clears any H_2S present in the circulation during a single pass at all but the highest reported physiological levels. A significant limitation of the isolated and perfused liver study is the absence of red blood cells. It has been suggested that H_2S may bind to hemoglobin or be metabolized by red blood cells into a physiologically inert form [138]. Therefore, we investigated the effect of H_2S infusion *in vivo* using intravital microscopy. Infusion of H_2S into the portal circulation via the splenic vein resulted in a significant decrease in hepatic oxygen content suggesting H_2S oxidation. Whether freely circulating or bound, our study demonstrates that hydrogen sulfide is rapidly cleared from the circulation during passage through the liver; however this clearance consumes available oxygen.

A recent study by Lagoutte et al. using isolated colonocytes demonstrated that H_2S is oxidized by the sulfide quininone reductase enzyme [68]. The study also demonstrated the capacity for H_2S oxidation from isolated mitochondria from several

different organs. Therefore, we sought to investigate whether the ability to metabolize hydrogen sulfide differed between organs. Using an isolated and perfused heart, we demonstrated that the heart has a very small capacity for H_2S oxidation, evidence that organ function and cellular environment contributes to the capacity for H_2S oxidation. Similar results were demonstrated in isolated and perfused kidneys and lungs [8]. Our results combined with those of Lagoutte et al indicate that the liver and colonic epithelial cells, cell types exposed to high levels of H_2S, are specialized for effective clearance of H_2S.

Lagoutte et al. also demonstrated that H_2S metabolism is a priority in mammalian cell types. Furthermore, oxidation of H_2S requires a significant amount of O_2 in a dose dependent manner. This finding was confirmed in our study. Low concentrations of H_2S (<200μM) resulted in a significant increase in oxygen consumption. As H_2S accumulated in the liver at higher doses a biphasic oxygen response was observed with an initial increase in oxygen consumption followed by a dramatic inhibition presumably from cytochrome oxidase c inhibition [87]. The initial increase in O_2 consumption at high doses of H_2S gradually decreased until an almost complete inhibition of O_2 consumption was observed at 500μM. It is possible that the increase in oxygen consumption observed during infusion of H_2S could be a result of the vasodilatory effect of H_2S. In order to assess the vasodilatory effect portal pressure was monitored throughout infusion of H_2S. Portal pressure remained stable throughout the entire experiment indicating that H_2S has little vasodilator contribution in the unstimulated isolated and perfused liver system (Data not shown).

Oxygen availability in the liver is subject to heterogeneous intralobular O_2 distribution which can be exacerbated during pathophysiological states such as sepsis [58]. Therefore, we tested whether alterations in O_2 availability affect H_2S metabolism. O_2 availability proved to be a requirement for H_2S metabolism as decreasing oxygen tension in the buffer resulted in a decreased ability to clear H_2S. In an extremely low O_2 environment the liver was almost incapable of clearing H_2S. Our findings are in agreement with the proposal that H_2S is a putative oxygen sensor as the levels of H_2S and O_2 are inversely related [91]. Moreover, a 2008 study by Whitfield et al demonstrated an accumulation of H_2S during periods of hypoxia that was rapidly reversed in the presence of oxygen [138]. Whether this increase in H_2S is beneficial or detrimental may rely on the specific location and stimulus of the increase. Where an increase in the vasculature would lead to the appropriate vasoactive response [93], an increase in H_2S in the liver may lead to tissue hypoxia and contribute to hepatic injury.

Due to its rapid oxidation in the liver, the possibility arises that H_2S is removed from circulation before it is able to exert a physiological effect. To test this possibility, isolated livers from fasted rats were stimulated with the alpha adrenergic agonist phenylephrine and gluconeogenic substrates, lactate and pyruvate. In agreement with other research groups, H_2S infusion resulted in an attenuation of the phenylephrine constrictor response indicating that H_2S was still biologically active [33]. Combined treatment with phenylephrine and H_2S led to greater O_2 consumption than phenylephrine alone. This was shown to be independent of gluconeogenesis as H_2S did not significantly alter phenylephrine-induced increases in gluconeogenesis, an oxygen dependent process [23]. Thus, the increase in oxygen consumption with co-treatment with phenylephrine

and H_2S is most likely a combination of increased hepatic perfusion as well as H_2S oxidation.

Hepatocellular dysfunction is known to occur in sepsis [19]. Loss of hepatocellular function leads to disease progression, however not all functions are affected equally [132]. Furthermore, hepatic oxygen availability during sepsis is decreased due to decreased hepatic perfusion resulting from an increased sensitization to vasoconstrictive mediators (e.g. ET-1) [12]. The decreased hepatocellular function combined with inadequate oxygen supply led us to hypothesize that the ability to metabolize H_2S would be greatly diminished in livers isolated from septic rats. Interestingly, our study demonstrated that metabolism of H_2S remains a priority in septic rat livers. One possible explanation could be the beneficial effect of constant flow perfusion on hepatic function. In an ischemia/reperfusion model, Chun et al demonstrated that perfusion at a constant flow maintained perfusion and prevented hepatocellular injury whereas constant pressure perfusion did not [22]. It is also important to note that these studies were done in the early stage of sepsis, prior to the development of septic shock. Our model did not produce any mortality within the 24 hour time period used; however, it is likely that some mortality would occur after several days. Importantly, our study demonstrates that the liver does not lose the capacity to metabolize H_2S during the early inflammatory phase of sepsis.

In summary, the present study demonstrates that the liver has an important role in the regulation of H_2S levels in the circulation. While the debate continues regarding physiologically relevant levels of H_2S, we provide evidence that the rapid oxidation of H_2S during a single passage through the liver maintains low systemic circulating levels of

H_2S. H_2S more likely acts as an auto- and paracrine signaling molecule [92]. However, especially during septic peritonitis, production of H_2S by intestinal bacteria is likely to increase H_2S levels in the hepatic portal blood. Excess H_2S production is removed from the circulation by the liver making it a key regulatory site for H_2S levels. The oxidation of increased levels of H_2S predisposes the liver to periods of tissue hypoxia. As hepatic O_2 levels drop, the capacity of the liver to metabolize H_2S decreases forming a detrimental positive feedback loop as more H_2S enters via the circulation. We have demonstrated that H_2S oxidation remains a priority during sepsis. H_2S oxidation could be especially detrimental during sepsis where increased H_2S levels during sepsis could exacerbate the limited oxygen available [13]. Tissue hypoxia contributes to hepatic injury through increased generation of reactive oxygen species [14, 83]. Additionally, HIF 1 alpha, a hypoxia-dependent signaling molecule, is pro-inflammatory and may contribute to hepatic injury [35, 75]. Ultimately, toxic levels of H_2S could lead to inhibition of cytochrome c oxidase. Recent studies have suggested a potential therapeutic value to an induced suspended state of animation of the cell brought about by H_2S presumably through cytochrome c oxidase inhibition [16]. Administration of H_2S has proven to provide therapeutic value in rodent models of ischemia/reperfusion injury [117], acetaminophen induced hepatotoxicity [84], and sepsis [117, 122, 144]. The complexity of H_2S during sepsis cannot be overstated as inhibition of H_2S production has proved therapeutic as well [144]. These discrepancies may be due to experimental parameters or time and dose of administration. Nevertheless, it is evident that H_2S has a role in pathophysiological states. We propose that the liver is unique in the role H_2S plays in

pathophysiological states due to the priority given to modulate hydrogen sulfide levels in the circulation at the expense of hepatic oxygen availability.

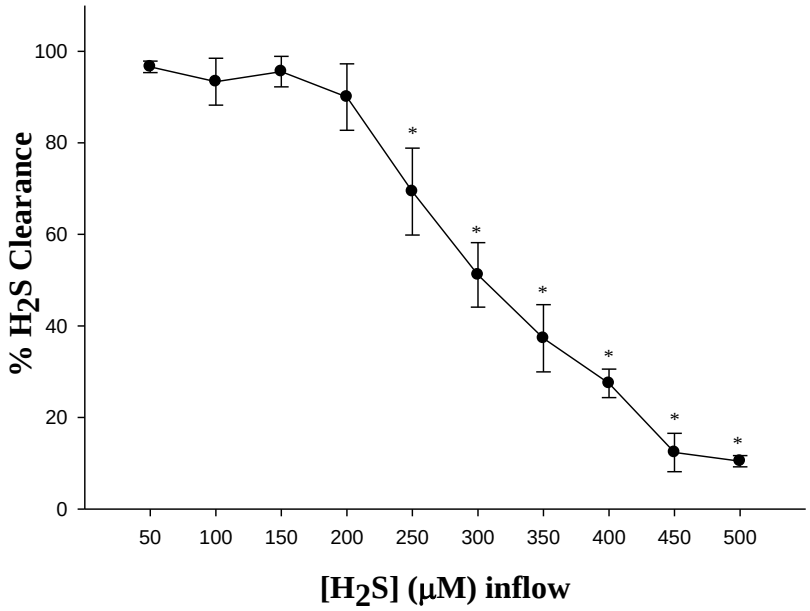

Figure 1: Percent removal of H_2S from perfusate by isolated and perfused liver**.** The liver was perfused *in situ* via the portal vein by a flow-controlled, non-recirculating perfusion system. The H_2S donor Na_2S was infused into the inflow perfusate to achieve final concentrations of 50-500 μM. Inflow and outflow perfusates were assayed for H_2S concentrations. The percent of H_2S cleared from perfusate is plotted against the calculated [H_2S] inflow. Data are presented as means ± SEM of four separate experiments (N=4). Statistical analysis performed using one way repeated measures ANOVA with Student-Newman-Keuls *post hoc* test. *P<0.001 versus 50 μM.

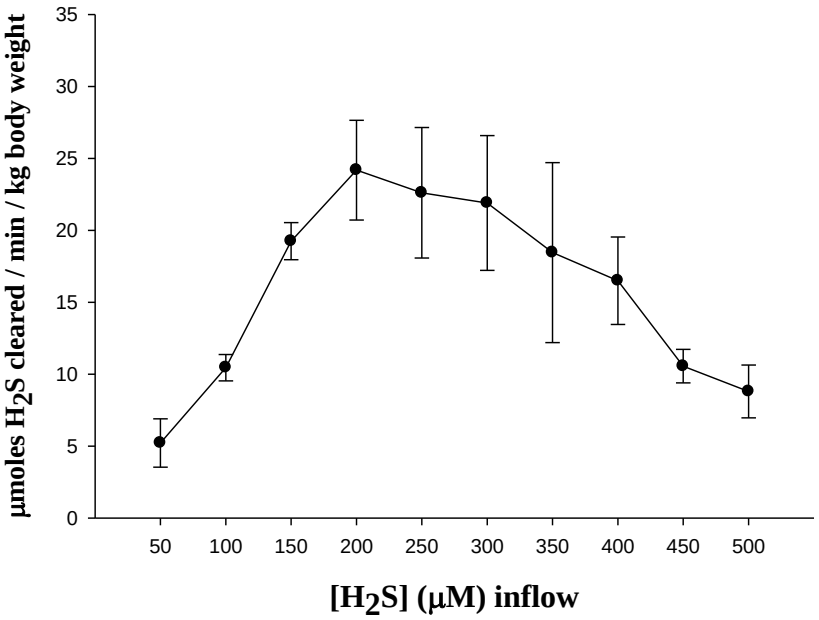

Figure 2: Total removal of H_2S from perfusate by isolated and perfused liver. The total amount of H_2S cleared was determined by calculating the amount of H_2S removed from the perfusate after a single pass through an isolated liver. Data are presented as means ± SEM of three separate experiments (N=3).

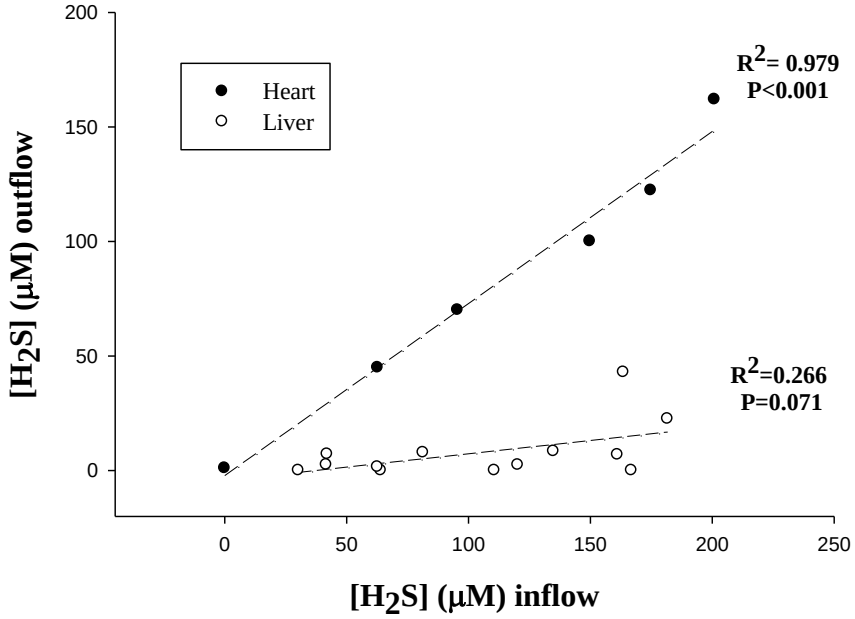

Figure 3: Isolated heart shows very limited capacity for H_2S clearance**.** An isolated and perfused heart was used for comparison to the liver's ability to clear H_2S from perfusate. Both organs were perfused at a constant flow rate with oxygenated Kreb's buffer. H_2S donor Na_2S was infused as 1% of total portal perfusion flow to achieve final perfusate concentrations of 50-200 μM. Inflow and outflow perfusate were assayed for H_2S concentration. Data are plotted as actual [H_2S] (μM) inflow versus actual [H_2S] (μM) outflow. Linear regression analysis was performed on final perfusate concentrations considered physiologically relevant (0-200μM).

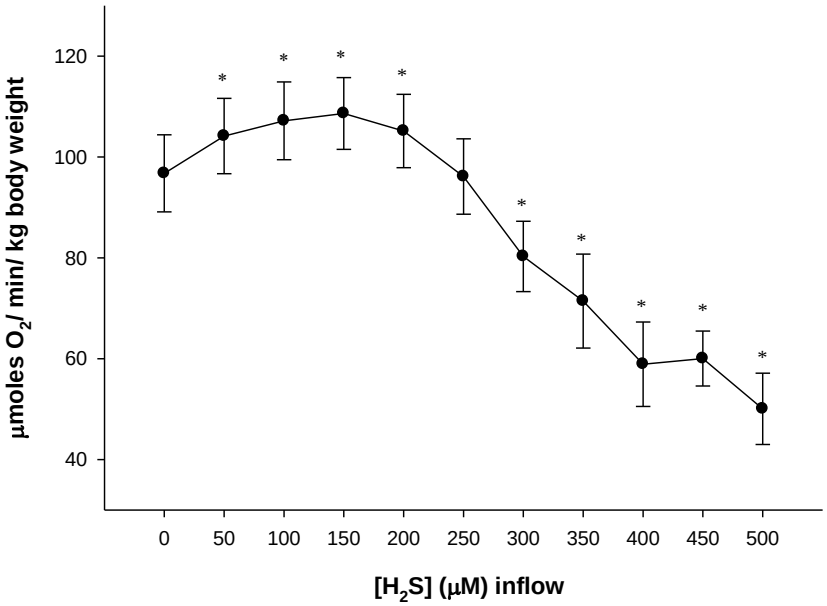

Figure 4: Hydrogen Sulfide metabolism produces a biphasic oxygen consumption response. Inflow and outflow perfusate oxygen tensions were monitored during infusion of increasing H_2S concentrations (0-500µM) using an oxygen sensitive electrode. The change in oxygen tension in the inflow versus outflow was calculated as the amount of µmoles of O_2 consumed per minute per kg of body weight. One way repeated measures ANOVA was used for statistical analysis. Analysis for physiological concentrations (0-200µM) and pathological concentrations (250-500µM) were performed independently due to the biphasic response. Data are represented as means ±SEM of three separate experiments (N=3). * $P<0.01$ compared to 0 µM infusion.

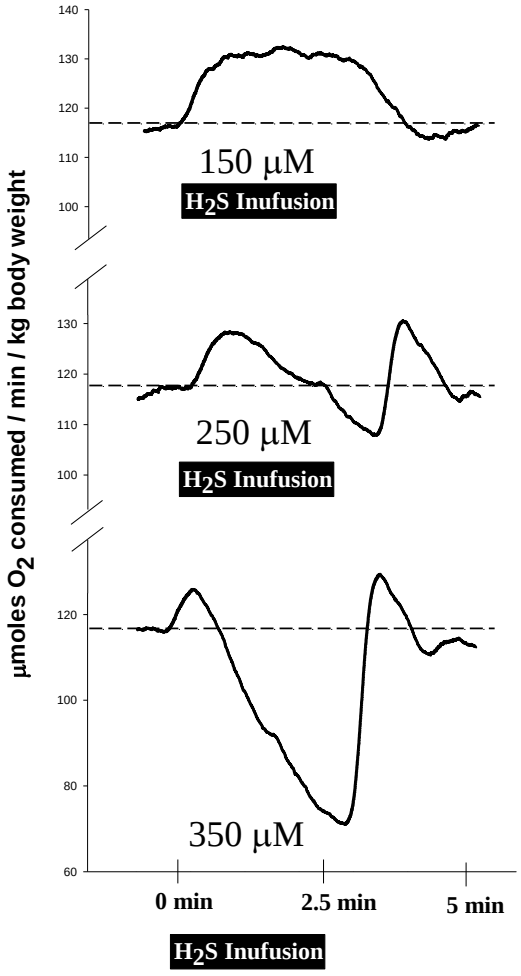

Figure 5: Individual tracings of biphasic oxygen consumption response to H_2S. Individual tracings of the 5 minute H_2S infusion/ recovery cycle observed at Na_2S perfusate concentrations of 150, 250, and 350µM. Na_2S was infused for a period of 2.5 minutes followed by a 2.5 recovery period. The dotted line indicates approximate baseline consumption for each tracing (118 µmoles O_2 consumed / min / kg body weight).

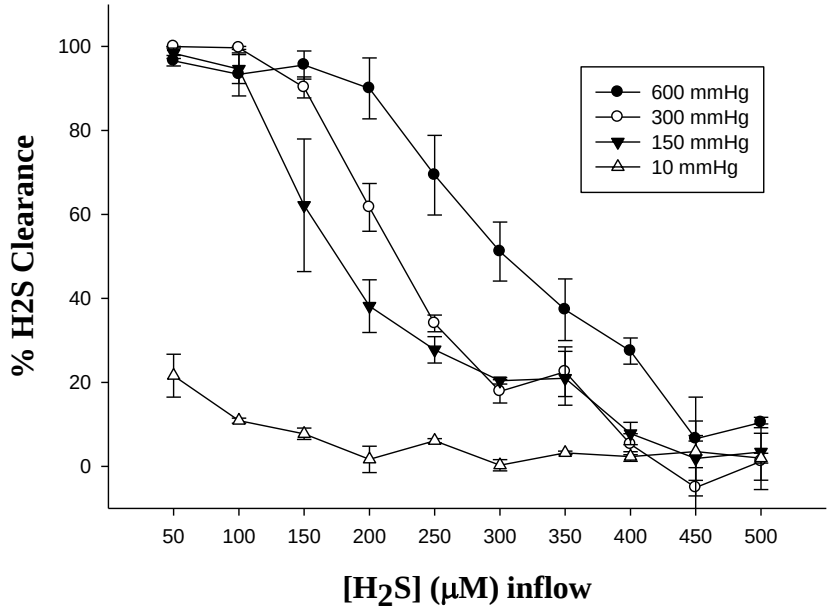

Figure 6: Oxygen availability is a requirement for H_2S metabolism at physiological concentrations**.** To produce differential oxygen availability in an isolated liver, Kreb's buffer was continuously bubbled with a mixture of O_2, N_2, and CO_2 to achieve approximate final inflow O_2 content of 600, 300, 150, and 10 mmHg. Inflow and outflow perfusate was collected and sampled for H_2S content. Results are plotted as % H_2S clearance versus calculated [H_2S] (μM) inflow. Data are presented as means ± SEM for three separate experiments (four in 600mmHg group) (N=3).

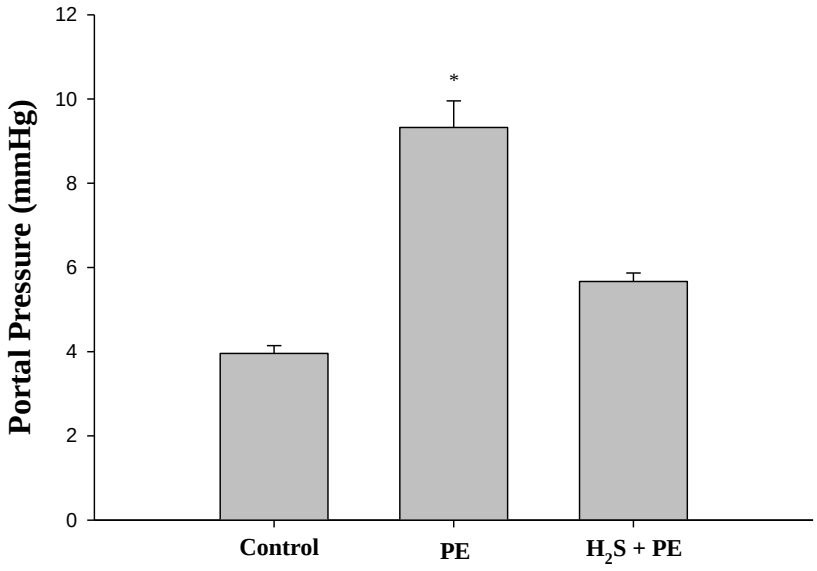

Figure 7: Hydrogen sulfide attenuates phenylephrine-induced increase in portal Pressure. Livers were isolated from rats fasted for 18 hours prior to surgery. Kreb's buffer alone was perfused for 20 min to allow for stabilization and baseline determinations followed by addition of phenylephrine (PE) with gluconeogenic substrates lactate (5mM) and pyruvate (1mM) in the presence or absence of 100 μM H_2S. Data are means ± SE of 4 PE experiments and 3 H_2S separate experiments (N=4,3). Statistical analysis performed using one way repeated measures ANOVA with Student-Newman-Keuls *post hoc* test. * $P<0.001$ compared to control.

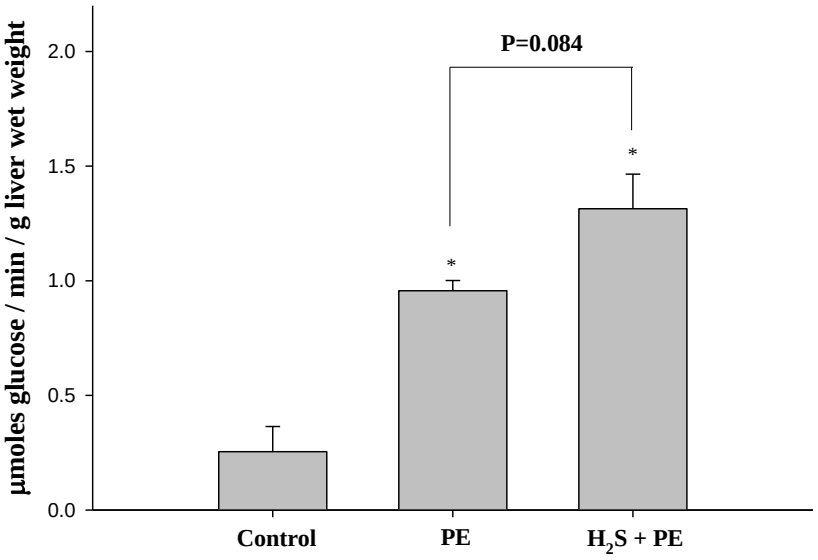

Figure 8: Hydrogen sulfide has no effect on stimulation of glucose production by Phenylephrine. Livers were isolated from rats fasted for 18 hours prior to surgery. Kreb's buffer alone was perfused for 20 min to allow for stabilization and baseline determinations followed by addition of phenylephrine (PE) with gluconeogenic substrates lactate (5mM) and pyruvate (1mM) in the presence or absence of 100 µM H_2S. Data are means ± SE of 4 PE experiments and 3 H_2S separate experiments (N=4,3). Statistical analysis performed using one way repeated measures ANOVA with Student-Newman-Keuls *post hoc* test. * $P<0.001$ compared to control. Outflow perfusate samples from isolated livers were collected for determination of glucose output.

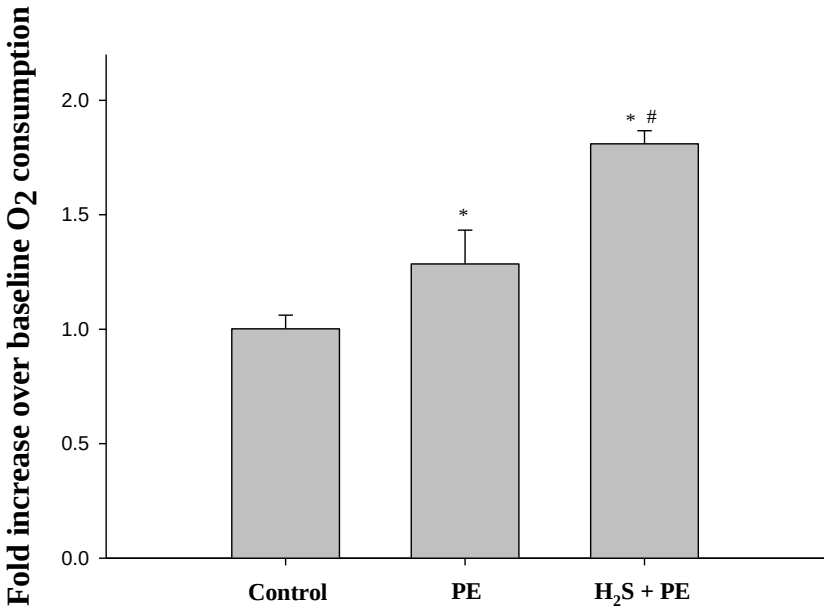

Figure 9: Hydrogen sulfide potentiates the increase in O_2 consumption following phenylephrine treatment. Livers were isolated from rats fasted for 18 hours prior to surgery. Kreb's buffer alone was perfused for 20 min to allow for stabilization and baseline determinations followed by addition of phenylephrine (PE) with gluconeogenic substrates lactate (5mM) and pyruvate (1mM) in the presence or absence of 100 μM H_2S. data are means ± SE of 4 PE experiments and 3 H_2S separate experiments (N=4,3). Statistical analysis performed using one way repeated measures ANOVA with Student-Newman-Keuls *post hoc* test. * $P<0.001$ compared to control. Oxygen levels in the outflow perfusate were monitored to determine the effect of H_2S and phenylephrine on oxygen consumption.

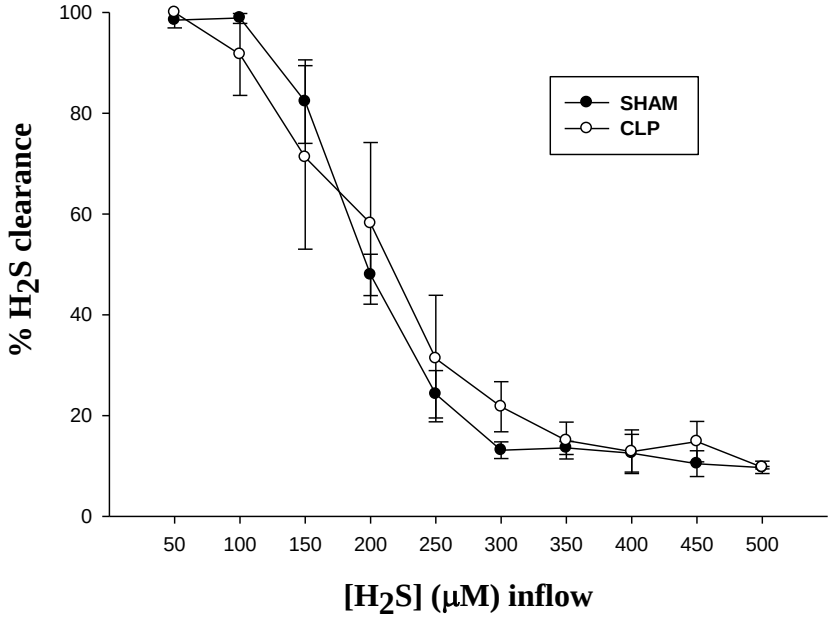

Figure 10: Cecal ligation and puncture does not affect percent hepatic H_2S clearance ability. Fasted rats underwent cecal ligation and puncture (CLP). Sham surgery served as a control. After 24 hours, livers were isolated and subjected to increasing concentrations of H_2S (50-500μM). Inflow and outflow perfusate were collected and assayed for H_2S content. Data are presented as the percentage of H_2S cleared in a single pass through the liver. Data are presented as means ± SEM of three separate experiments (N=3).

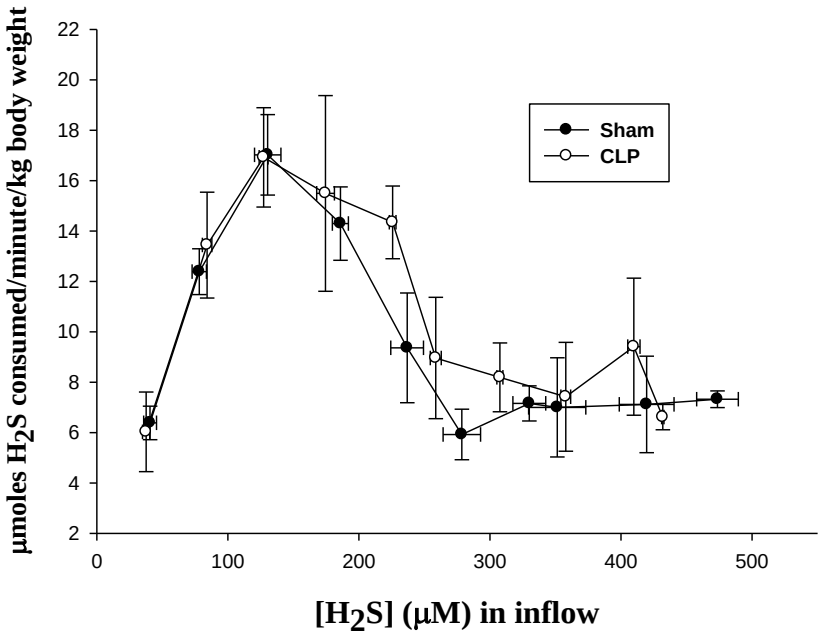

Figure 11: Cecal ligation and puncture does not affect total hepatic H_2S clearance ability**.** Total H_2S clearance was measured from livers isolated from CLP and sham animals following infusion of Na_2S resulting in H_2S perfusate concentrations between 50-500µM. The total amount of H_2S cleared is plotted against estimated inflow [H_2S]. Data are presented as means ± SEM of three separate experiments (N=3).

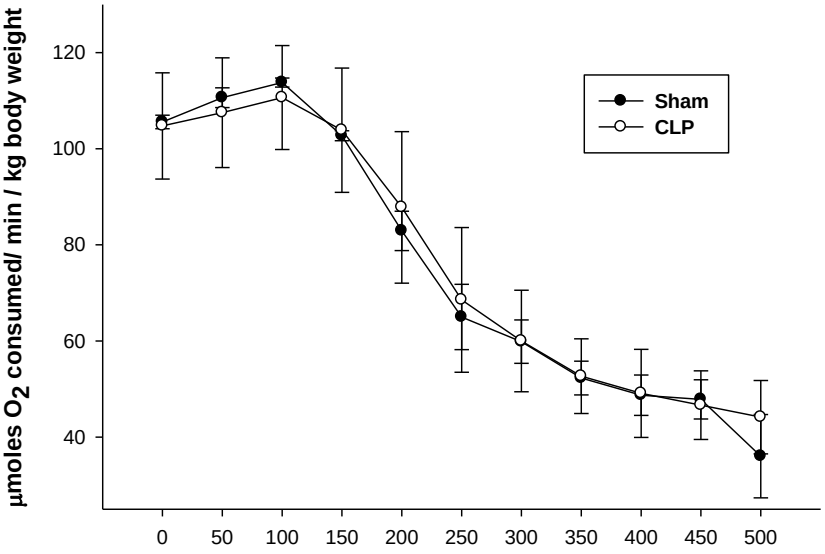

Figure 12: Cecal ligation and puncture does not affect hepatic O_2 consumption. Outflow perfusate from isolated livers from Sham and CLP treated rats was continuously monitored with an oxygen sensitive probe to determine the effect of H_2S on O_2 consumption. Data are presented as means ± SE of three separate experiments (N=3).

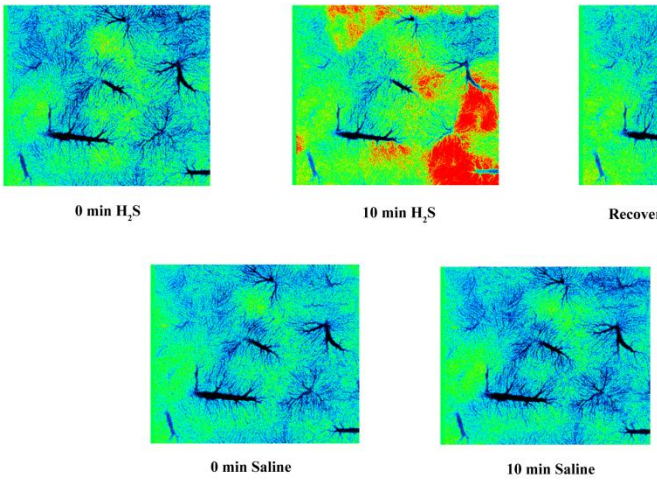

Figure 13: H_2S infusion is associated with an increase in NADH autofluorescence. Intravital microscopy was used to assess oxygen distribution in the hepatic microenvironment. 10 min saline infusion demonstrated stable oxygen distribution before H_2S infusion. H_2S was infused into the portal circulation for 10 minutes followed by a 10 minute saline infusion recovery period. NADH fluorescence (excitation 366nm, emission 450nm) increases as the ratio of $NADH/NAD^+$ increases and is an indirect measure of oxygen availability. Gray scale was converted to pseudocolor with blue representing black (low $NADH/NAD^+$) and red representing white (high $NADH/NAD^+$).

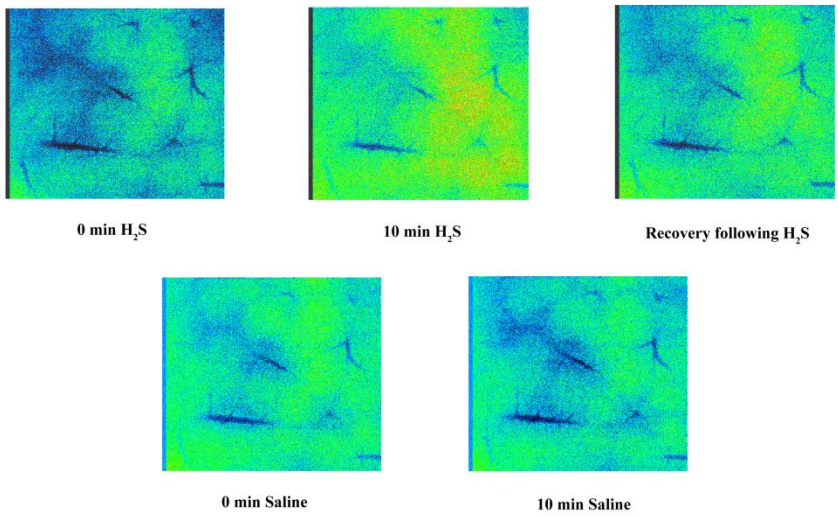

Figure 14: H_2S infusion is associated with a decrease in hepatic O_2 content. Intravital microscopy was used to assess oxygen distribution in the hepatic microenvironment. 10 min saline infusion demonstrated stable oxygen distribution before H_2S infusion. H_2S was infused into the portal circulation for 10 minutes followed by a 10 minute saline infusion recovery period. $Ru(Phen)_3^{2+}$ was infused into the circulation to serve as a direct oxygen sensor using intravital microscopy. $Ru(Phen)_3^{2+}$ fluorescence (excitation 480 nm, emission 625nm) increases in the absence of oxygen. Grayscale was converted to pseudocolor with blue representing black (high O_2) and red representing white (low O_2). Micrographs (4x objective) of one experiment are representative of three individual experiments (N=3).

CHAPTER 3: HYDROGEN SULFIDE DIFFERENTIALLY AFFECTS THE HEPATIC VASCULATURE IN RESPONSE TO PHENYLEPHRINE AND ENDOTHELIN-1 DURING ENDOTOXEMIA

3.1 Abstract

Despite being protective in many disease states, H_2S contributes to organ injury in sepsis. Like the other gasotransmitters, nitric oxide (NO) and carbon monoxide (CO), H_2S is a modulator of the microcirculation. Since microcirculatory dysfunction is a main cause of organ injury during sepsis, the present study was designed to test the effect of H_2S on microvascular dysfunction in isolated, perfused livers. In most microcirculatory beds, endotoxin activates the endothelium resulting in hyporesponsiveness to catecholamines and a derangement in blood flow distribution. We demonstrate that H_2S treatment attenuates the increase in portal pressure during infusion of the $\alpha_1$adrenergic agonist, phenylephrine (PE) ($P<0.01$). H_2S almost completely negated the increase in portal pressure in livers isolated from endotoxemic rats. Treatment with an inhibitor of endogenous H_2S, dl-propargylglycine (PAG), reversed LPS-induced hyporesponsiveness to PE. Since hepatic microcirculatory dysfunction is associated with excessive sinusoidal vasoconstriction and not dilation, we investigated whether H_2S affects ET-1-induced vasoconstriction in isolated livers. Contrary to PE treatment, H_2S did not affect the increase in portal pressure during infusion of ET-1 nor did it attenuate the hypersensitization of the liver to ET-1 during endotoxemia. Hepatic resistance in control rats was increased by PAG treatment during ET-1 infusion, but this increase was not

exacerbated during endotoxemia. We monitored hepatic O_2 consumption to assess the effect of vascular changes on oxygen consumption following ET-1 treatment. Low dose ET-1 infusion caused an increase in hepatic O_2 consumption, whereas low dose ET-1 infusion decreased O_2 consumption in endotoxemic livers. Interestingly, while we observed no effect of PAG on the vascular response to ET-1 infusion during endotoxemia, PAG treatment did maintain O_2 suggesting a more complex effect of H_2S inhibition. In summary, the discrepancies between the hepatic response to PE and ET-1 suggest that H_2S differentially contributes to microcirculatory dysfunction in the systemic and hepatic microcirculations. We propose that this is due to H_2S exerting a differential vasoactive function on presinusoidal and sinusoidal sites within the liver. Moreover, our findings suggest that H_2S may contribute to the progression of sepsis by contributing to microvascular failure.

3.2 Introduction

Organ failure is a common complication during sepsis and is associated with an increase in morbidity and mortality [78]. Microcirculatory dysfunction is an important contributor to organ injury [52]. In most vascular beds, sepsis attenuates vasoconstriction in response to catecholamine signaling. This contributes to intractable hypotension, inadequate tissue perfusion, and organ dysfunction. The portal venous circulation is also hyporesponsive to catecholamine signaling; however, depressed catecholamine signaling does not contribute to hepatic microvascular dysfunction [97]. Instead, sinusoidal hyperconstriction, due to increased sensitivity to the vasopressor, endothelin-1 (ET-1), results in heterogeneous perfusion and focal hypoxia which contributes to hepatic dysfunction and ultimately overall injury [12-13, 94].

H_2S is primarily produced as a byproduct of cysteine metabolism by two pyridoxal 5' phosphate (PLP) dependent enzymes: cystathionine β synthase (CBS) and cystathionine γ lyase (CSE) [18, 123]. Additionally, a third PLP independent enzyme, 3 mercaptopyruvate sulphurtransferase (3MST) synthesizes H_2S [114]. CSE is the predominant source of H_2S in the cardiovascular system and liver [123]. The liver is likely exposed to elevated levels of H_2S during bacterial peritonitis due to an increase in hepatic CSE expression and the arrival of H_2S in the portal circulation synthesized from by bacteria of the GI tract [144].

There are conflicting reports in the literature about the vasoregulatory action of H_2S [4, 48, 74, 140, 148]. The first indication that H_2S modulates the hepatic vasculature was reported by Fiorucci et al. Using an isolated perfused liver system, they demonstrated that H_2S attenuated the increase in portal pressure during infusion of norepinephrine (NE) in normal and cirrhotic livers [33]. Therefore, H_2S may be beneficial during cirrhosis and fibrosis by lowering intrahepatic resistance and reducing portal hypertension. Based on this finding, one would predict that elevated levels of H_2S would prevent sinusoidal constriction during sepsis; however, there is ample evidence demonstrating that hepatic dysfunction during sepsis is the result of sinusoidal constriction resulting in tissue hypoxia [12-13, 63, 88].

The hepatic microcirculation has multiple sites of blood flow regulation. Upstream of the sinusoids, terminal portal venules are surrounded by VSMCs and respond to vasoactive molecules similarly to other vascular beds. Unlike the capillaries in other vascular beds, the hepatic sinusoids can modulate local tissue perfusion by changing their resistance which makes them a second, functionally important site of

hepatic perfusion [146]. Unlike portal venules, the sinusoids lack VSMCs. Instead, they are surrounded by hepatic stellate cells (HSC) which can modulate perfusion through individual sinusoids by altering their contractility in response to vasoactive molecules [49, 62]. This unique organization allows regulation of overall hepatic perfusion by pre-sinusoidal regulatory and spatial distribution of hepatic blood flow within the liver lobule [57, 99]. Previous work from our lab has demonstrated that the α_1 adrenergic agonist, phenylephrine (PE) and ET-1 both increase hepatic vascular resistance [146]. Importantly, PE acts only on pre-sinusoidal sites of regulation, whereas ET-1 acts on pre-sinusoidal and sinusoidal regulatory sites [10-11, 146]. Inflammatory stress primes the liver to the vasoconstrictive effect of ET-1 resulting in sinusoidal hyperconstriction and tissue hypoxia [13, 107].

Therefore, the present study was designed to investigate the effect of H_2S on the different sites in hepatic microvasculature during endotoxemia. Using an isolated perfused liver system we investigated the effect of H_2S on total intrahepatic resistance during portal infusion of PE or ET-1 during endotoxemia. We hypothesize that H_2S acts as a vasodilator in response to PE, but has no effect on ET-1-induced vasoconstriction, particularly during endotoxemia. This study is the first to demonstrate that H_2S differentially regulates pre-sinusoidal and sinusoidal sites of hepatic perfusion. Furthermore, our finding that H_2S contributes to systemic vascular hyporesponsiveness, but does not affect sinusoidal constriction adds to the current knowledge regarding the detrimental effects of H_2S on in organ injury during sepsis [144].

3.3 Materials and Methods

Animals**:** Fifty Male Sprague-Dawley rats (Charles River Laboratories, Fayetteville, NC) weighing 272±13.2 g were used in this study. Rats remained quarantined for four days following arrival. Rats were maintained in pairs in standard cages with bedding with free access to standard rat chow and water in a temperature-controlled setting under 12-hour light/dark cycles. Rats were removed from the vivarium on the morning of the experiment and transported to the laboratory where all surgical procedures were performed. Animals were randomly assigned to groups on the day of the experiment. All animal manipulation was in strict adherence with National Institutes of Health guidelines and experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of North Carolina at Charlotte.

Materials: Sodium sulfide (Na_2S), L-phenylephrine (PE), lipopolysaccharide (LPS, Escherichia coli O26:B6), DL-propargylglycine (PAG), were purchased from Sigma-Aldrich Co. (St. Louis, MO). Endothelin-1 (ET1) was purchased from American Peptide Company (Sunnyvale, CA).

Isolated Perfused Liver: Isolated, perfused rat livers were used to investigate the effect of vasoactive modulators on hepatic portal resistance as described previously [125] with modification [89]. Briefly, rats were anesthetized under isoflurane and subjected to laparotomy. Heparin (1 USP unit/rat mass (g), Webster Veterinary, Devens, MA) was injected into the inferior vena cava. The portal vein was exposed and three loosely tied sutures were placed along the length of the vein. The portal vein was cannulated and the sutures were tightened to secure the cannula in place. The inferior vena cava and abdominal aorta were severed to prevent hypervolemia due to perfusion and to allow for

exsanguation. The pleural cavity was opened and a cannula was inserted into the right atrium and directed downward in the superior vena cava. The cannula was secured in place just below the diaphragm. Lastly, the inferior vena cava was ligated above the renal veins to block perfusate flow. This isolates the liver *in situ* during perfusion with oxygenated Kreb's Henseleit buffer. Temperature of the buffer was maintained at 37° Celsius and continuously oxygenated using 95% O_2/5% CO_2 gas. Following a 20 minute stabilization period, increasing cumulative doses of PE (0.05-20 μM) or ET-1 (0.05-1.0 nM) were infused sequentially in 5 minute intervals. Total intrahepatic resistance was determined by monitoring the pressure in the inflow tubing (portal pressure) using a Biopac Systems MP100 transducer (Goleta, CA) during constant flow perfusion (16ml/kg/min). The H_2S donor Na_2S (50 μM) was administered in the perfusate during ET-1 or PE infusions for certain animal groups. In other experiments, the CSE-inhibitor propargylglycine (PAG, 1 mg/kg) was injected intraperitoneally 30 minutes before liver isolation to inhibit endogenous H_2S production. For the endotoxin model of sepsis, rats were given an injection of LPS (1 mg/kg, ip) 6 hours before liver isolation and perfusion.

Oxygen Monitoring: The amount of O_2 extracted by the liver from the perfusate was calculated to assess hepatic oxygen consumption. The perfusate PO_2 were monitored using an oxygen sensitive Clark-type electrode connected to an oxygen monitor (YSI Life Sciences, Yellow Springs, Ohio). All measurements were recorded continuously using Biopac systems MP 100 transducer and software (Goleta, CA). Calibration of the electrode was performed on the daily using distilled water equilibrated with air and adjusted for temperature and elevation. The concentration of the inflow perfusate was measured was measured immediately prior to and after the experiment. O_2 extraction was

calculated by as the difference between the inflow perfusate PO_2 and outflow perfusate PO_2.

Statistical Analysis: The data are presented as means ± standard error (SEM). Statistical significance was determined using 2 way ANOVA analysis. Student-Newman-Keuls *post hoc* test was used when ANOVA analysis detected significance. Independent and repeated measures analysis was used where appropriate. Statistical significance was $P < 0.05$.

3.4 Results

Effect of endotoxin on hepatic resistance in response to PE and ET-1**:** An isolated, perfused liver system was utilized to determine the effect of PE and ET-1 on the hepatic vasculature during endotoxemia. Baseline portal pressure in control rats was 3.2±1.4 mmHg. Despite being slightly elevated, there was not a statistically significant effect of 6 hour LPS treatment (4.5±1.6 mmHg, P=0.076). PE infusion produced an increase in portal pressure with a maximal increase of 5.5±0.8 mmHg with infusion of 20μM PE in control animals (Figure 15). LPS treatment significantly attenuated the increase in portal pressure due to PE infusion with a maximal increase in portal pressure of 4.1±1.0 mmHg (P<0.001, Figure 15). LPS treatment had the opposite effect on the vasculature response to ET-infusion. LPS potentiated the increase in portal pressure during ET-1 infusion (P<0.001, Figure 16). Additionally, LPS caused a significantly greater maximal increase in portal pressure (15.2±1.1 mmHg in control vs. 18.7±2.3 mmHg in LPS, P<0.017).

Effect of H_2S on hepatic resistance in response to PE and ET-1**:** To determine the effects of hydrogen sulfide on hepatic resistance, isolated livers were perfused with Kreb's buffer or Kreb's buffer containing the H_2S donor Na_2S (50 μM). H_2S did not

significantly affect the resting portal pressure (4.1±2.7 mmHg vs. 3.2±1.4 mmHg in controls, P=NS). H_2S treatment significantly reduced the dose dependent increases in portal pressure during PE infusions (Figure 17, P=0.01). H_2S had no effect on the increase in portal pressure during infusion of ET-1 (Figure 18).

Effect of combined H_2S/LPS treatment on hepatic resistance in response to PE and ET-1**:** LPS treatment followed by perfusion with 50 μM H_2S Kreb's buffer did not affect the resting portal pressure (4.2±0.5 mmHg). Infusion of 5μM PE increased portal pressure 5.4±0.5 mmHg (Figure 19). LPS and H_2S each reduced this response to 3.3±0.5 and 3.1±.4 mmHg respectively (P<0.05). A synergistic interaction between LPS and H_2S produced a nearly complete attenuation of the increase in portal pressure during 5μM PE infusion (1.6±0.5 mmHg increase in H_2S/LPS P<0.05). Infusion of 0.5 nM ET-1 increased portal pressure by 13.6±1.0 mmHg in control rats (Figure 20). This effect was significantly increased in LPS-treated rats (19.3±1.3 mmHg increase, P<0.01). H_2S had no effect on the increase in portal pressure in neither control nor LPS-treated rats (15.0±1.5 and 18.2±1.5 mmHg respectively).

Effect of inhibition of endogenous H_2S synthesis on hepatic resistance during endotoxemia: Hepatic synthesis of H_2S was inhibited by treating rats with dl-propargylglycine (PAG) (50mg/kg ip) 30 minutes prior to liver isolation. PAG treatment alone had no effect on the resting portal pressure when compared to controls (3.6±0.9 mmHg vs. 3.2±1.4 mmHg). PAG treatment caused a greater increase in portal pressure due to infusion of PE (P<0.001, Figure 21) and ET-1 (P<0.001, Figure 22) when compared to control animals. To test the effect of endogenous H_2S synthesis during endotoxemia, rats were treated for 6 hrs with LPS then given PAG 30 minutes prior to

isolation. The combination of PAG and LPS had no effect on baseline portal pressure when compared to controls (4.8±0.7 mmHg). Infusion of 5 μM PE resulted in an increase in portal pressure of 4.7±0.5 mmHg in controls which was reduced to 3.3±0.6 mmHg in LPS-treated rats ($P<0.05$, Figure 23). PAG treatment reversed the effect of LPS on PE-induced vasoconstriction during endotoxemia (6.1±0.5 mmHg, $P<0.05$). An 11.3±0.8 mmHg increase in portal pressure was observed during infusion of 0.5 nM ET-1 in control rats (Figure 24). LPS and PAG treatment both caused a greater increase in portal pressure to ET-1 (16.2±1.1 and 15.4±1.3 mmHg respectively, $P<0.05$). The combination of PAG and LPS was not significantly different from LPS or PAG alone (14.7±1.3 mmHg).

Effect of H_2S and PAG on O_2 consumption during ET-1 infusion. There was no difference in resting hepatic O_2 consumption between any of the treatment groups prior to ET-1 infusion. For livers in H_2S treatment groups, the addition of 50 μM Na_2S to the perfusate increased O_2 consumption (7% over baseline, $P<0.05$) which was used as a baseline for these groups. In control livers, infusion of 0.05 nM ET-1 produced a modest, but repeatable increase in O_2 consumption (6% increase over baseline, $P<0.05$). In livers isolated from LPS-treated rats, infusion of 0.05 nM demonstrated a trend towards decreased oxygen consumption, but it was not statistically significant (100% vs. 92 %, $P=0.058$). However, hepatic O_2 consumption during infusion of 0.05 nM ET-1 was significantly lower in the LPS group when compared to controls (90 % vs. 106 %, $P<0.05$). The H_2S group and H_2S/LPS group were not significantly different from either the control or LPS groups during infusion of 0.05 nM ET-1 (Figure 25, 99% of baseline and 98% of baseline, respectively). PAG treatment was significantly lower than controls

during 0.05 nM ET-1 infusion ($P<0.05$, Figure 26). While PAG appeared to restore O_2 consumption in LPS-treated animals, the results were not statistically significant ($P=0.055$). When the concentration of ET-1 was increased to 1 nM, hepatic O_2 consumption was significantly reduced in all treatment groups ($P<0.001$). 2 way ANOVA analysis demonstrated a significant effect of H_2S on hepatic O_2 consumption which was increased over control and LPS treatment groups. PAG and PAG/LPS treatment groups were reduced during infusion of 1 nM ET-1 similar to control and LPS groups and no significant differences were observed.

3.5 Discussion

The role of H_2S in disease and inflammation is the source of considerable debate. H_2S prevents myocardial and hepatic ischemia/reperfusion injury which is partially the result of an increase in cellular antioxidant capacity via induction of antioxidant gene expression [59, 117]. On the contrary, inhibition of endogenous H_2S synthesis significantly reduces organ injury and improves survival in septic mice by attenuating the inflammatory response [72, 144]. In the earliest stages of sepsis, hepatocellular injury is initially the result of excessive cytokine release from kupffer cells (KCs) [6, 134]; however, it is now well established that continued hepatocellular dysfunction is primarily the result of hepatic microvascular failure, particularly in the sinusoids [12-13, 63, 94]. As a vasoregulatory molecule that is elevated in the liver during sepsis [144], it is possible that H_2S contributes to the dysregulation of hepatic sinusoidal perfusion.

Fiorucci et al were the first to demonstrate a vasoregulatory role of H2S in the hepatic microcirculation. In their study, H_2S attenuated the increase in intrahepatic resistance to norepinephrine (NE) in normal and cirrhotic rats. This vasodilatory effect

would likely be beneficial during chronic conditions, like cirrhosis, by decreasing intrahepatic resistance and attenuating portal hypertension [72]. If H_2S acts as a vasodilator in the hepatic microcirculation, one would predict that H_2S could also be beneficial during sepsis via improved tissue perfusion. The hepatic microcirculation is regulated at presinusoidal and sinusoidal sites [90]. Therefore, we tested the effect of H_2S on the vascular response to phenylephrine and endothelin-1 that act at these sites, respectively, in an endotoxin model of sepsis. Our study demonstrates that H_2S differentially modulates the response of the hepatic microcirculation to vasopressor which suggests that H_2S may exert a different vasoregulatory function at different sites within the liver. Moreover, our results highlight the complex role H_2S serves in modulating the microcirculation during endotoxemia.

Several factors contribute to systemic microcirculatory dysfunction during sepsis, including activation of pro-coagulant pathways, recruitment of leucocytes, and excess production of vasoactive agents [52]. In healthy individuals, the activation of α1 adrenergic receptors by circulating catecholamines causes vascular smooth muscle cell contraction and reduces blood flow through the vascular bed which allows for the spatial distribution of cardiac output. During sepsis, the excessive release of vasodilators suppresses the response of the endothelium to catecholamines [97]. The diffuse peripheral vasodilation contributes to systemic hypotension and organ dysfunction [47].

In this study, we first investigated the effect of H_2S on pre-sinusoidal portal venules which respond to catecholamines in a similar manner to systemic resistance vessels. Evidence to support this was provided by studies demonstrating decreased vascular responsiveness to phenylephrine in livers isolated from endotoxemic rats.

Moreover, administration of an inhibitor of NOS reversed the hyporesponsive of the hepatic vasculature suggesting the importance of increased production of vasodilatory gas NO in vascular hyporesponsiveness [97]. Like NO, H_2S acts as a vasodilator which suggests that a similar effect of H_2S should occur during endotoxemia [48]. Infusion of increasing doses of PE produced a dose dependent increase in portal pressure in isolated livers, which was significantly diminished in livers isolated from LPS-treated rats. The addition of exogenous H_2S (50μM) to the perfusate almost completely abrogated the increase in portal pressure in response to PE in livers isolated from endotoxemic rats suggesting a synergistic effect of LPS and H_2S.

We used the suicide inhibitor of CSE to block hepatic H_2S synthesis during endotoxemia to determine if endogenous H_2S production contributes to vascular hyporesponsiveness. PAG treatment alone significantly potentiated the increase in intrahepatic resistance in response to PE, suggesting that H_2S is involved in the constant regulation of hepatic blood flow. Importantly, PAG treatment reversed the hyporesponsiveness of the presinusoidal venules to PE during endotoxemia.

Given that the only target of PE in the hepatic microcirculation are pre-sinusoidal resistance vessels [146], the previous results suggest that H_2S is involved in the modulation of hepatic microvasculature at the level of portal terminal venules. Because the liver is the largest internal organ and a major recipient of cardiac output, excessive hepatic vasodilation could potentially contribute to systemic hypotension during sepsis. H_2S derived from CSE is an important regulator of systemic blood pressure [140]. Since portal venules and systemic arterioles are regulated in a similar manner by vascular smooth muscle cells (VSMCs), it is likely that H_2S contributes to hyporesponsiveness of

resistance vessels in most tissues. In support of this hypothesis, circulating H_2S levels in septic rats have been shown to be negatively correlated to blood pressure [50].

Based on our results, PAG treatment may provide a protective effect during sepsis by attenuating systemic vascular hyporesponsiveness to catecholamines; however, its effect in the hepatic sinusoid remains unclear. Previously, we showed that sinusoidal tone is modulated by hepatic stellate cells (HSCs) which contract in response to ET-1 [146]. HSCs are activated following inflammatory stress which enhances their contractility to ET-1 [107]. The priming effect of LPS on the HSCs results in sinusoidal hyperconstriction which is a main cause of tissue hypoxia and cell death [13]. In support of the importance of ET-1 in hepatic dysfunction, elevated ET-1 levels in are highly correlated to disease severity in cirrhosis [3]. Thus, the sinusoid is a critical regulatory site for hepatic perfusion during sepsis. Previous work from our lab and others demonstrated that impaired synthesis of NO contributes to sinusoidal dysfunction [67, 81, 98]. The vasoregulatory effect of H_2S in the hepatic sinusoids remains unclear. Therefore, we investigated the effect of H_2S on the sinusoids which during endotoxemia by assessing its effect on ET-1 infusion.

Infusion of increasing concentrations of ET-1 resulted in progressive increase in portal pressure. In agreement with our previous reports, the effect of ET-1 was significantly potentiated by endotoxin treatment. Based on its vasodilatory action, we hypothesized that H_2S would attenuate the hypersensitization of the hepatic sinusoid to ET-1 during endotoxemia. Surprisingly, we observed no effect of H_2S on ET-1-induced vasoconstriction in neither control nor endotoxin treated rats. Interestingly, PAG treatment increased the vascular response to ET-1 in control livers, but had no effect in

the endotoxin group. These results suggest that H_2S differentially modulates the vascular response to ET-1 when compared to PE.

This differential effect may be due to the cells responsible for modulating lumenal diameter. Portal terminal venules, as well as arterioles, are modulated by VSMCs. The vasodilatory effect of H_2S is primarily the result of VSMC hyperpolarization via activation of K_{ATP} channels [103, 128, 148]. Hepatic sinusoidal resistance is regulated by HSCs which may be differentially regulated by H_2S. It has been suggested that the H_2S donor (NaHS) can prevent HSC contraction [30]. However, that study was performed in isolated HSCs over the course of 18 hours with NaHS. The spontaneous activation of HSCs following isolation may not reflect actual *in vivo* conditions [130]. Furthermore, NaHS rapidly releases H_2S which if not contained in a closed system rapidly escapes culture media and enters the atmosphere in a short period of time during incubation [71]. While the finding of that study is promising, more conclusive research is needed to assess the effect of H_2S on HSCs.

One drawback of our isolated, perfused liver system is that we are assessing total intrahepatic resistance by monitoring changing in pressure during constant flow perfusion. An anticipated consequence of this is that the vasoconstriction at one location can be counteracted by vasodilation at another location. The suppression of a response during PE clearly demonstrates a vasodilatory effect of H_2S; however the lack of an effect during ET-1 infusion may be due to a set of more complex vascular events. It is possible that H_2S can induce vasoconstriction in the hepatic sinusoid which lack VSMCs and vasodilation at presinusoidal sites.

While H_2S is generally regarded as a vasodilator, there are several reports that it can function as a vasoconstrictor. In VSMCs, H_2S has been shown to lower cAMP levels and inhibit contraction [74]. H_2S could increase the sensitivity to ET-1 by lowering cAMP levels in HSCs. cAMP has been shown to desensitize ET_A receptors on HSCs to ET-1 [104]. A second mechanism, reported by Ali et al, demonstrated that high doses of exogenous H_2S produce hypotension, whereas low dose intravenous infusion of the H_2S donor, NaSH, produced a transient increase in mean arterial pressure in rats [4]. Interestingly, no increase in MAP was observed following treatment with L-NAME, a NOS inhibitor suggesting an interaction between the two gasses. It has been proposed that H_2S may interact with NO to form a vasoinactive nitrosthiol thereby quenching the nitric oxide signal [137]. This could be of particular interest in endotoxemia as ET-1 stimulated NO synthesis in SECs is diminished [67]. It is possible in our study that the addition of exogenous H_2S may quench NO availability causing constriction in the sinusoids while also hyperpolarizing VSMCs in the terminal portal venules resulting in not net change in intrahepatic resistance.

Previously, we reported that H_2S potentiates the increase in O_2 consumption induced by PE infusion which may contribute to hypoxic stress [89]. ET-1 stimulates glycongenolysis and O_2 consumption in isolated rat hepatocytes [111, 146]. Therefore, we investigated the effect of H_2S and PAG on ET-1 stimulated O_2 consumption. Infusion of low concentrations of ET-1, which did not produce a vascular response, increase hepatic O_2 consumption in control livers. At higher doses of ET-1, O_2 consumption was significantly reduced to the excessive vasoconstriction of the sinusoids. Hepatic O_2 consumption was significantly reduced by LPS treatment most likely due to enhanced

sinusoidal vasoconstriction. This finding highlights the importance of sinusoidal integrity in maintaining proper O_2 delivery during endotoxemia. The addition of H_2S to the perfusate caused an initial increase in O_2 consumption due to hepatic oxidation of H_2S. While H_2S had no effect in control or LPS livers during 0.5 nM ET-1 infusion, it was associated with a significant increase in O_2 consumption at the highest dose of ET-1. Throughout the experiment, H_2S was undetectable in outflow perfusate indicating complete hepatic H_2S oxidation. Despite the decreased O_2 consumption during sinusoidal hyperconstriction, the liver still utilized O_2 for H_2S oxidation providing further evidence that H_2S oxidation may contribute to hypoxic stress during endotoxemia. We observed that PAG potentiated the vascular effect of ET-1, in agreement with this hepatic O_2 consumption was significantly reduced compared to controls during low dose infusion of ET-1. While the PAG results in LPS-treated animals were not statistically significant (P=.055), there was a trend toward restoring hepatic O_2 consumption to baseline levels. Since the reduction in O_2 consumption in LPS-treated animals is the result of sinusoidal constriction it is tempting to speculate that PAG treatment may improve sinusoidal perfusion during endotoxemia.

The present study sought to investigate the effect of H_2S on different regulatory sites in the hepatic microcirculation. Using an isolated, perfused organ system, we demonstrated a differential effect of H_2S on sites presinusoidal resistance vessels that respond to PE and the sinusoids which are modulated primarily by ET-1. We conclude that H_2S contributes to the loss of vasomotor control in resistance vessels subject to regulation via catecholamine signaling. While we cannot conclude that H_2S causes vasoconstriction in the sinusoids, we do demonstrate that there is no attenuation of ET-1

hypersensitization, suggesting differential regulation in the sinusoids. Additionally, we show that hepatic oxidation occurs despite limited O_2 availability due to sinusoidal hyperconstriction. This finding combined with our previous report that H_2S lowers hepatic oxygen availability *in vivo* supports the hypothesis that elevated H_2S levels may exacerbate hepatic tissue hypoxia. The discrepancy between the effects of H_2S on PE and ET-1 demonstrate the need for a better understanding of H_2S as a vascular modulator. Overall, our study demonstrates that the regulation of hepatic vascular tone by H_2S is complex and likely an important modulator in microvascular dysfunction during endotoxemia.

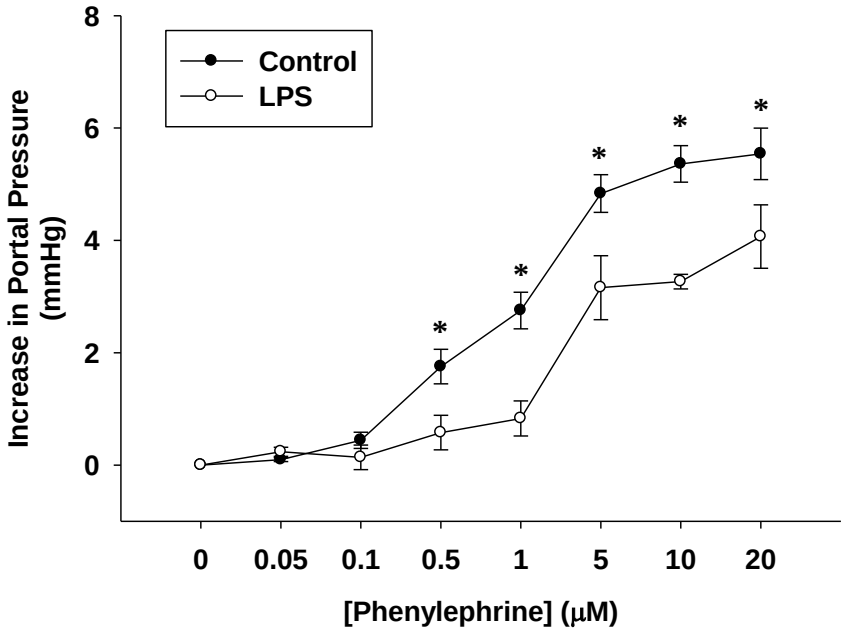

Figure 15: LPS attenuates the hepatic vascular response to phenylephrine**.** Isolated, perfused livers from control rats or rats treated for 6 hours with LPS (1mg/kg ip) were subjected to increasing doses of PE. Portal pressure was monitored during constant flow perfusion with Kreb's buffer. LPS attenuated the dose dependent increase in portal pressure caused by PE ($P<0.001$). Data is presented as the mean increase in portal pressure over baseline ± SEM and compared using two way ANOVA with SNK *post hoc* test. N=4 in control group, N=5 in LPS group.

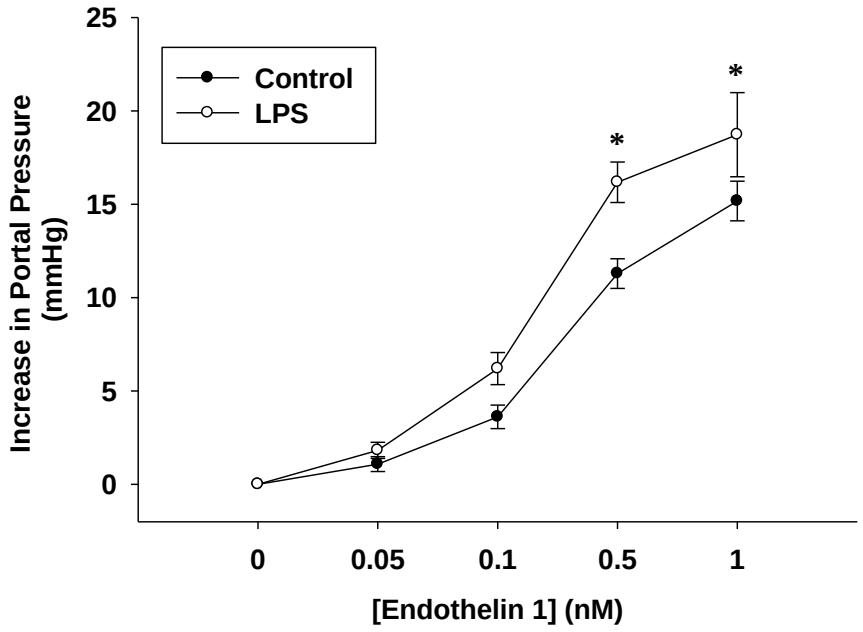

Figure 16: LPS potentiates the hepatic vascular response to endothelin-1. Isolated, perfused livers from control rats or rats treated for 6 hours with LPS (1mg/kg ip) were subjected to increasing doses of ET-1. Portal pressure was monitored during constant flow perfusion with Kreb's buffer. LPS potentiates the increase in portal pressure caused by ET-1 ($P<0.001$). Data is presented as the mean increase in portal pressure over baseline ± SEM and compared using two way ANOVA with SNK *post hoc* test. N=7 in control group, N=4 in H_2S group.

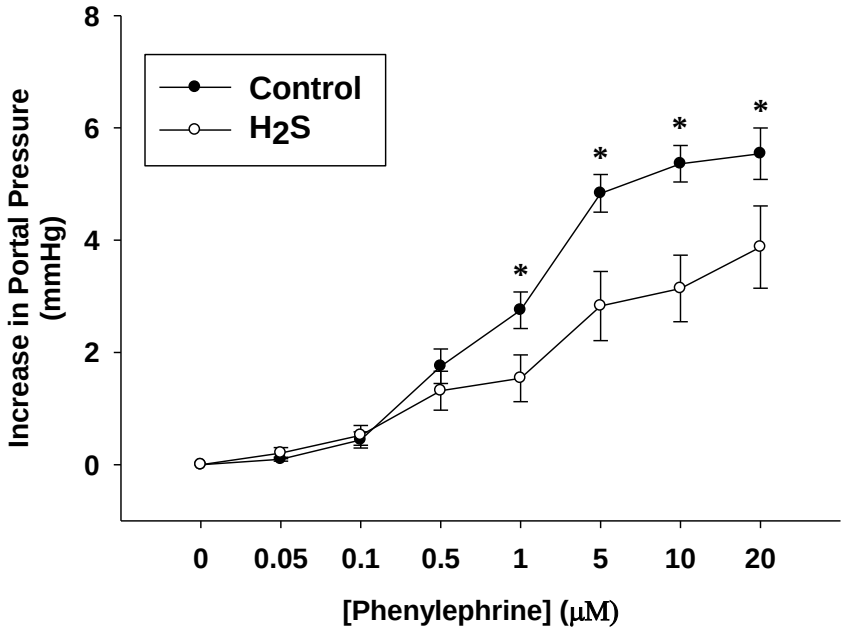

Figure 17: H_2S attenuates the increase in portal pressure caused by PE. Isolated, perfused livers were perfused at a constant flow rate with control buffer or buffer containing 50 μM Na_2S. H_2S attenuates PE-induced increases in portal pressure ($P>0.001$). Data is presented as the mean increase in portal pressure over baseline ± SEM and compared using two way ANOVA with SNK *post hoc* test. N=4 in control group, N=6 in H_2S group.

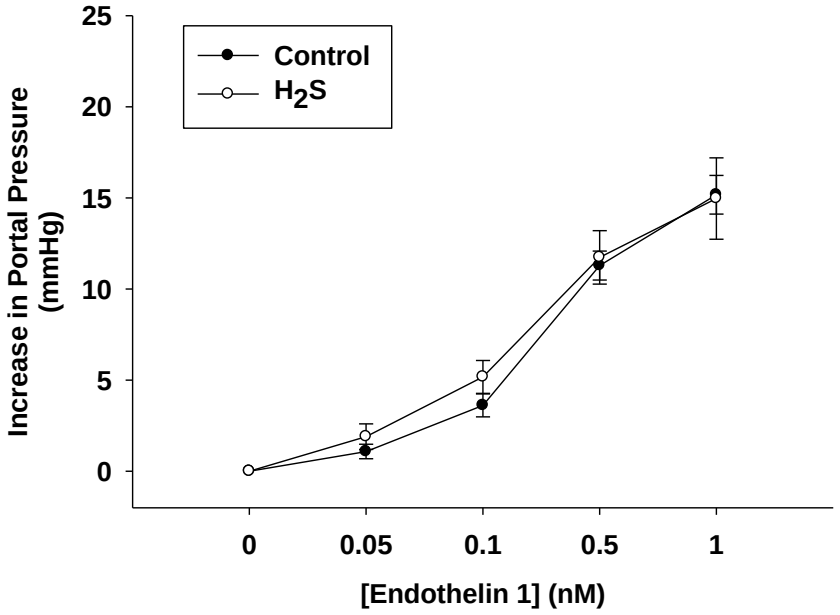

Figure 18: H_2S has no effect on the increase in portal pressure caused by ET-1. Isolated, perfused livers were perfused at a constant flow rate with control buffer or buffer containing 50 μM Na_2S. H_2S has no effect on ET-1 mediated increases in portal pressure (P=NS). Data is presented as the mean increase in portal pressure over baseline ± SEM (3-6 rats per group) and compared using two way ANOVA with SNK *post hoc* test. N=7 in control group, N=3 in H_2S group.

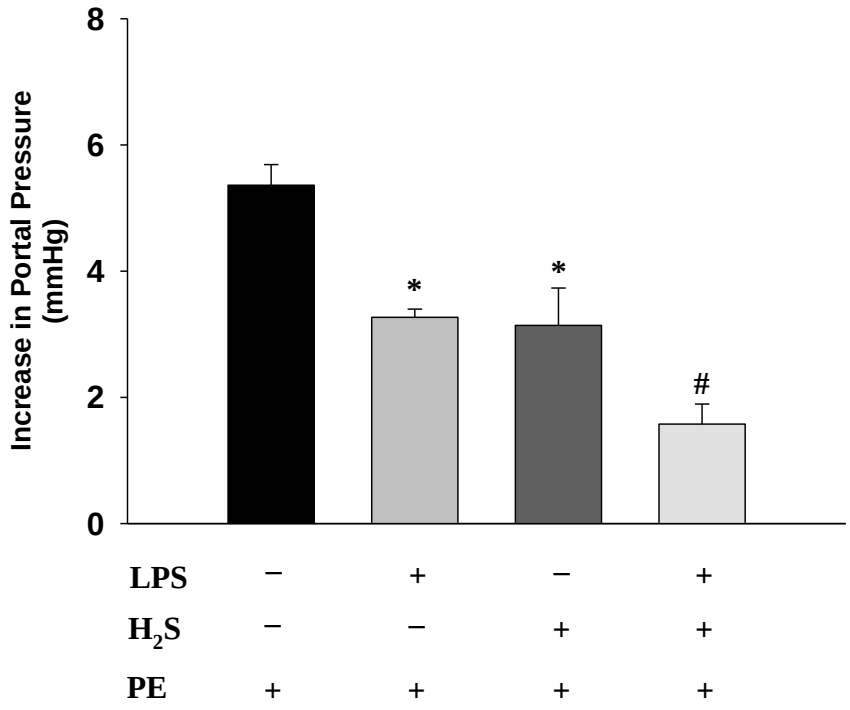

Figure 19: H_2S potentiates LPS-induced vascular hypo-responsiveness to PE. Livers isolated from control and endotoxemic rats were perfused with control or 50 μM H_2S Kreb's buffer. Graph shows mean increase in portal pressure (mmHg) during infusion of 10 μM PE. Data is presented as mean ± SEM and compared using two way ANOVA with SNK *post hoc* test. * $P<0.05$ compared to control. # $P<0.05$ compared to LPS and H_2S. N=4 in control group, N=5 in LPS and H_2S/LPS group, N=6 in H_2S group.

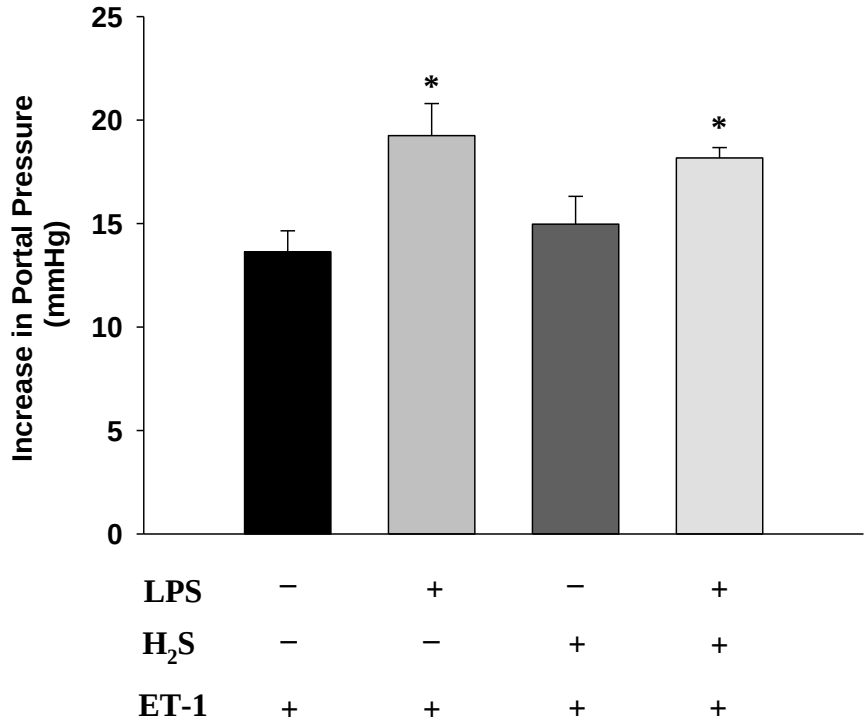

Figure 20: H_2S does not affect LPS-induced vascular hyper-responsiveness to ET-1. Livers isolated from control and endotoxemic rats were perfused with control Kreb's buffer. Graph shows mean increase in portal pressure (mmHg) during infusion of .5 nM ET-1. Data is presented as mean ± SEM and compared using two way ANOVA with SNK *post hoc* test. * $P<0.05$ compared to control. N=7 in control group, N=4 in LPS group, N=3 in H_2S and H_2S/LPS groups.

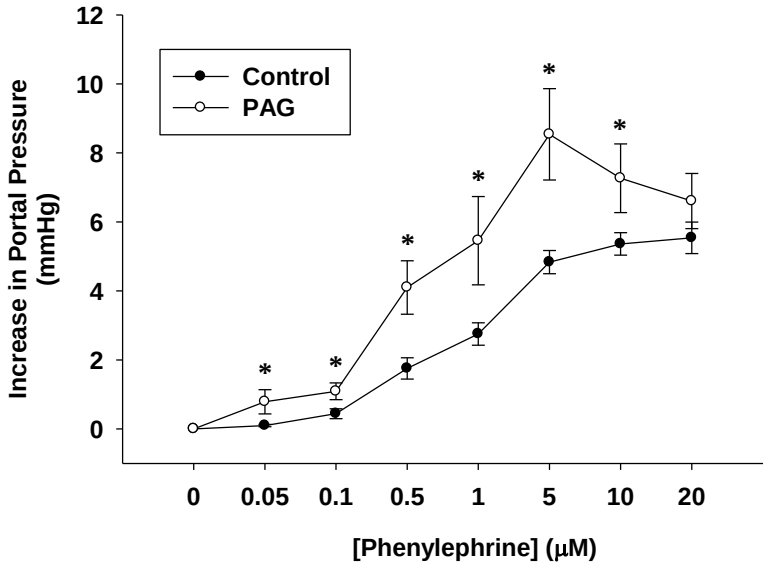

Figure 21: PAG treatment increases sensitivity of the hepatic vasculature to PE. 30 minutes prior to liver isolation rats were treated with CSE inhibitor PAG (50mg/kg ip). PAG potentiates the increase in portal pressure during infusion of PE ($P<0.001$). Data is presented as mean ± SEM and compared using two way ANOVA with SNK *post hoc* test. N=4 in control group, N=3 in PAG group.

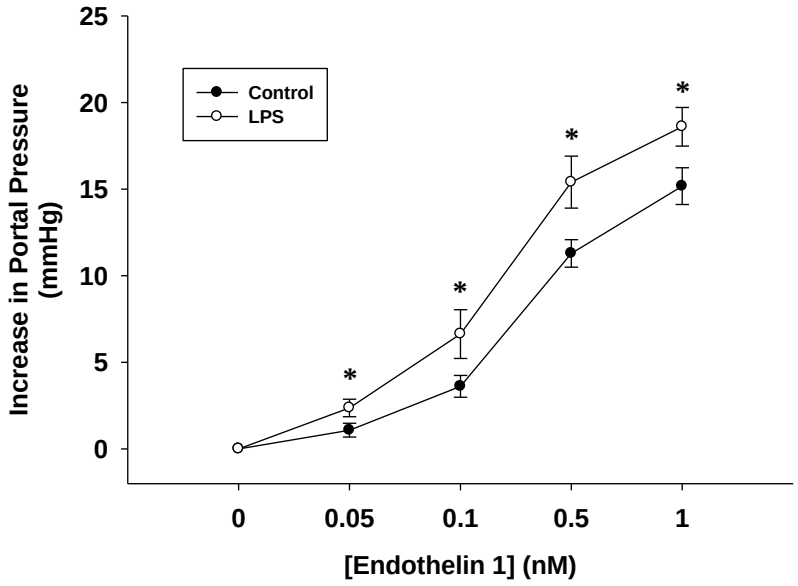

Figure 22: PAG treatment increases sensitivity of the hepatic vasculature to ET-1. 30 minutes prior to liver isolation rats were treated with CSE inhibitor PAG (50mg/kg ip). PAG potentiates the increase in portal pressure during infusion of ET-1 ($P<0.001$). Data is presented as mean ± SEM and compared using two way ANOVA with SNK *post hoc* test. N=7 in control group, N=3 in PAG group

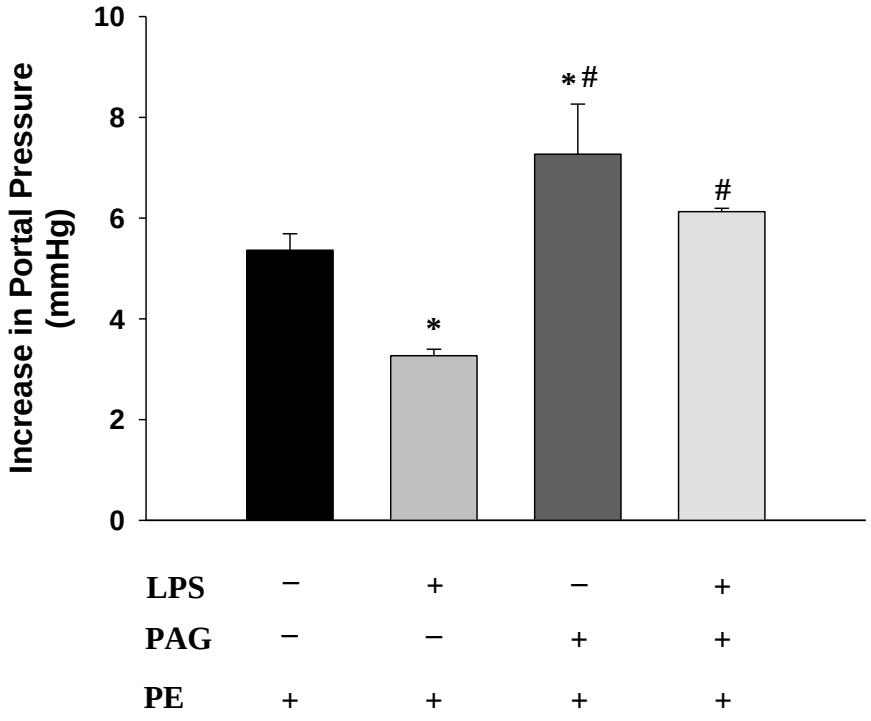

Figure 23: PAG treatment reverses hepatic vascular hypo-responsiveness to PE during endotoxemia**.** Control and endotoxemic rats were treated with PAG (50mg/kg) 30 minutes prior to liver isolation. Mean increase in portal pressure (mmHg) during infusion of 10 μM PE. Data is presented as mean ± SEM and compared using two way ANOVA with SNK *post hoc* test. * $P<0.05$ compared to control. # $P<0.05$ compared to LPS. N=4 in controls and LPS group, N=3 in PAG and PAG/LPS groups

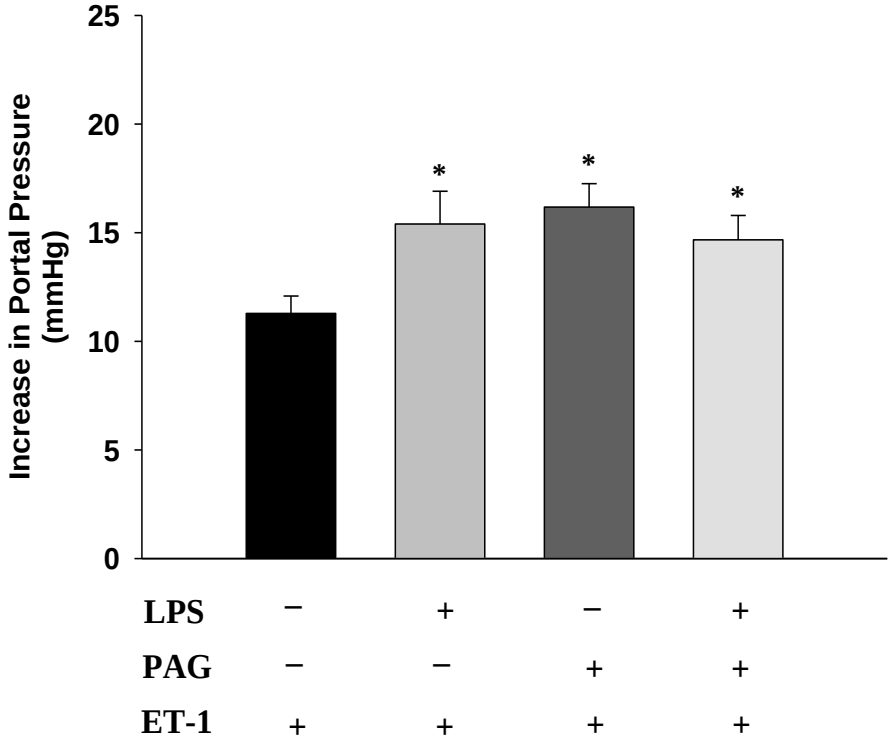

Figure 24: PAG does not potentiate the hepatic vascular response to ET-1 during endotoxemia**.** Control and endotoxemic rats were treated with PAG (50mg/kg) 30 minutes prior to liver isolation. Mean increase in portal pressure (mmHg) during infusion of 0.5 nM ET-1. Data is presented as mean ± SEM and compared using two way ANOVA with SNK *post hoc* test. * $P<0.05$ compared to control. N=7 in control group, N=4 in LPS group, N=3 in PAG and PAG/LPS group.

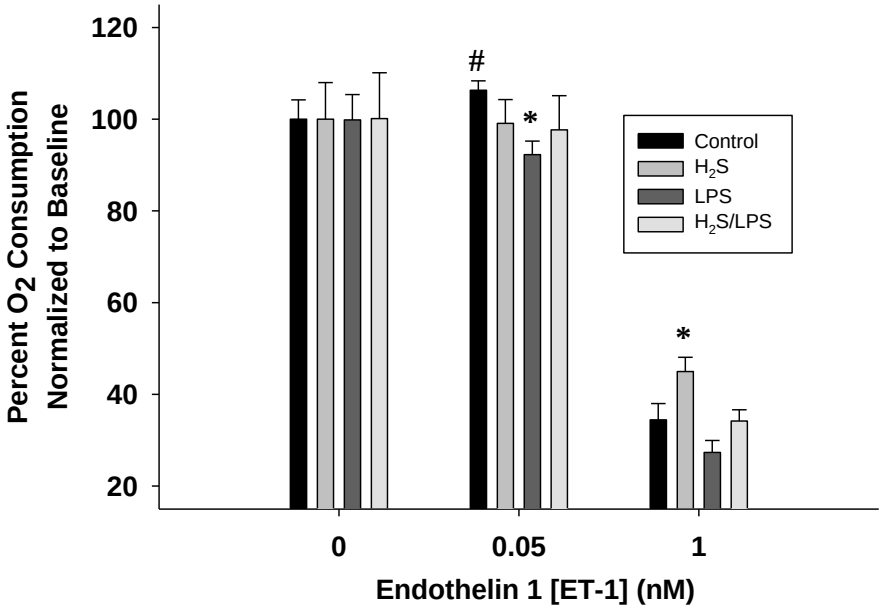

Figure 25: Effect of H_2S on hepatic O_2 consumption in control and endotoxemic livers. The PO_2 of the outflow and inflow perfusate was monitored to determine hepatic O_2 consumption during ET-1 infusion. The effect of H_2S on hepatic O_2 consumption during endotoxemia. Data is presented as mean ± SEM and compared using two way ANOVA with SNK *post hoc* test. * $P<0.05$ compared to control. # $P<0.05$ compared to baseline within each treatment group.

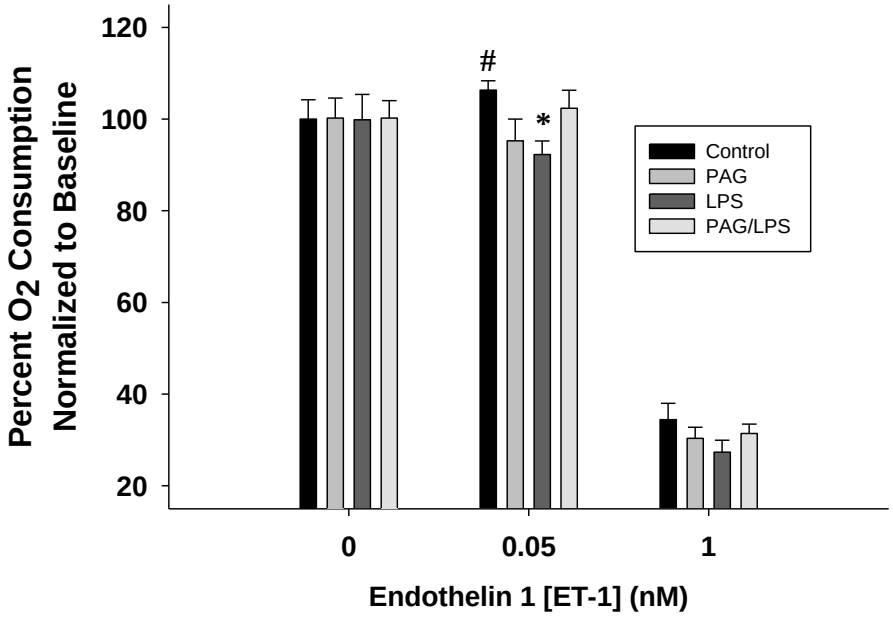

Figure 26: Effect of PAG on hepatic O_2 consumption in control and endotoxemic livers. The PO_2 of the outflow and inflow perfusate was monitored to determine hepatic O_2 consumption during ET-1 infusion. The effect of PAG on hepatic O_2 consumption during endotoxemia. Data is presented as mean ± SEM and compared using two way ANOVA with SNK *post hoc* test. * $P<0.05$ compared to control. # $P<0.05$ compared to baseline within each treatment group.

CHAPTER 4: HYDROGEN SULFIDE MODULATES SINUSOIDAL CONSTRICTION AND CONTRIBUTES TO HEPATIC MICORCIRCULATORY DYSFUNCTION DURING ENDOTOXEMIA

4.1 Abstract

Hydrogen sulfide (H_2S), is a mediator in the regulation of vascular resistance; however, its effect on the hepatic microcirculation has yet to be investigated. The unique regulation of hepatic microvascular resistance is perturbed during sepsis which contributes to liver injury. Therefore, the present study was designed to determine the effect of H_2S on the hepatic microcirculation and to determine the contribution of endogenous H_2S to hepatic microcirculatory dysfunction in an endotoxin model of sepsis. We show that portal infusion of H_2S is associated with an increase in portal pressure (6.8 ± 0.2 mmHg before H_2S vs. 8.6 ± 0.8 mmHg peak during H_2S infusion, $P<0.05$). Using *intravital* microscopy, we observed a reduction in sinusoidal diameter (6.2 ± 0.27 μm before H_2S vs. 5.7 ± 0.3 μm after H_2S, $P<0.05$) and an increase in sinusoidal heterogeneity during H_2S infusion ($P<.05$) suggesting that H_2S produces vasoconstriction of the hepatic sinusoid. Since hepatic H_2S levels are elevated during sepsis, we used the cystathionine γ lyase inhibitor (CSE), dl-propargylglycine (PAG), to determine the contribution of H_2S to the hypersensitization of the sinusoid to the vasoconstrictive effect of endothelin-1 (ET-1). Our results show that PAG treatment significantly attenuated the hepatic sinusoidal sensitization to ET-1 in endotoxin treated animals. ET-1 infusion increased portal pressure to 175% of baseline in endotoxemic animals which was reduced

to 143% following PAG treatment ($P<0.05$). We show that PAG abrogated the increase in sinusoidal constriction after ET-1 infusion in LPS treated rats (30.9% reduction in LPS rats vs. 11.6% in PAG/LPS rats, $P<0.05$). Moreover, PAG treatment significantly attenuated the increase in NADH fluorescence following ET-1 exposure during endotoxemia (61 grey scale units LPS vs. 21 units in PAG/LPS, $P<0.05$) suggesting an improvement in hepatic oxygen availability. This study is the first to demonstrate a vasoconstrictive action of H_2S on the hepatic sinusoid and provides a possible mechanism for the protective effect of PAG treatment reported during sepsis.

4.2 Introduction

Perturbed hepatic sinusoidal perfusion is a critical factor in the development of focal tissue hypoxia leading to liver injury during sepsis [12-13, 63]. Sinusoidal blood flow is regulated by alterations of vascular tone by extra-sinusoidal resistance vessels, consisting of terminal portal venules and hepatic arterioles, and by the sinusoids themselves [146]. During sepsis, the excessive systemic production of vasodilatory agents, in particular nitric oxide (NO), leads to hyporesponsiveness of the extra-sinusoidal resistance vessels to the constrictor effect of catecholamines which can contribute to diffuse hypotension [97]. However, a local detrimental effect on the liver results from sinusoidal hyperconstriction and heterogeneous perfusion during sepsis [13].

Hepatic sinusoidal tone is regulated mainly by hepatic stellate cells (HSCs) which surround individual sinusoids [146]. These contractile pericytes respond to a local balance between vasoactive mediators to modulate sinusoidal tone [62]. It is now well recognized that liver injury, such as in fibrosis or cirrhosis, leads to HSC transdifferentiation of a myofibroblastic phenotype resulting in an increased contraction

in response to the vasoactive peptide endothelin-1(ET-1) [107]. However, increased HSC contractile response to ET-1 is also observed following a relatively mild inflammatory stress such as with low-dose endotoxemia or sepsis [13, 94]. Under normal conditions, ET-1 stimulated NO production in sinusoidal endothelial cells (SECs) mitigates the constrictor effect of ET-1 [98]. However, inflammatory stress impairs eNOS derived NO production in the sinusoidal endothelial cells [67]. The constrictor effect of ET-1 is not counteracted by the dilator effect of NO, producing sinusoidal constriction and microvascular dysfunction. Furthermore, exacerbation of ET-1 mediated sinusoidal constriction during sepsis is associated with an increase in heterogeneous sinusoidal perfusion, resulting in a mismatch between O_2 supply and cellular demand [13, 57]. Inefficient O_2 delivery results in the development of focal hypoxia and hepatic injury. While it is evident that NO is a significant mediator in sinusoidal tone, it is likely that it is not the only contributor to microvascular dysfunction given the plethora of other vasoactive molecules present in the hepatic microenvironment. Moreover, although exogenous NO donors can restore hepatic microcirculatory function in experimental models [41], a beneficial effect remains elusive in clinical trials [77, 121]. Therefore, more research regarding the regulation of sinusoidal perfusion is needed to identify other potential therapeutic targets.

The discovery that hydrogen sulfide (H_2S) is endogenously synthesized in mammals revived interest into the noxious gas typically associated with the smell of rotten eggs [135]. Studies show that hepatic H_2S synthesis is increased during sepsis and endotoxemia [26, 144]. Like NO, H_2S acts as a vasodilator [48]. Whereas NO-induced vasodilation is cGMP dependent [51], the primary mechanism of vasodilation induced by

H_2S is via hyperpolarization of VSMCs [148]. The effect of H_2S in the liver vasculature was first reported by Fiorucci et al. H_2S significantly attenuated the increase in intrahepatic resistance during norepinephrine (NE) infusion in isolated livers from normal and cirrhotic rats [33]. The vasodilatory effect of H_2S is likely beneficial during cirrhosis by increasing hepatic perfusion and reducing portal hypertension. Based on this finding, one would predict that as a vasodilator, H_2S would improve hepatic tissue perfusion and provide a benefit during sepsis. However, numerous studies have shown that endogenous H_2S contributes to disease progression in sepsis [5, 72, 144].

Recently, we reported that H_2S attenuates the pressor effect of phenylephrine (PE) in an isolated and perfused liver which is in agreement with the study by Fiorucci et al; however, we also showed that H_2S has no effect on ET-1-induced increases in portal pressure using the same model. Similar to NE, phenylephrine (PE) is known to act on extra-sinusoidal resistance vessels only, while ET-1 acts on extra-sinusoidal and sinusoidal sites of blood flow regulation [146]. The vasodilatory effect of H_2S during PE infusion is likely the result of VSMC hyperpolarization in the portal venules. However, sinusoidal tone in response to ET-1 is modulated by HSCs, not VSMCs. The finding that H_2S affects PE, but not ET-1 pressor effects, suggests that the vasoactivity of H_2S may be different in the hepatic sinusoid compared to the portal venules. Therefore, the present study was designed to investigate the vasoregulatory effect of H_2S on the hepatic sinusoids *in vivo*. Since H_2S has been shown to be deleterious during sepsis, we hypothesized that endogenous H_2S contributes to the hypersensitization of the hepatic sinusoid to ET-1 during sepsis. Using *intravital* microscopy, we show for the first time that infusion of endogenous H_2S is associated with net sinusoidal constriction and

increased perfusion heterogeneity in normal liver. Moreover, our study demonstrates that inhibition of endogenous H_2S production significantly reduces hepatic microvascular dysfunction in response to ET-1 during endotoxemia which leads to improved O_2 delivery to the tissue. Taken together, our results challenge the notion that H_2S solely acts as a vasodilator in the liver. Rather, the role of H_2S is likely fundamentally different in the hepatic sinusoid than it is in most resistance vessels, including the portal venules. Furthermore, we provide one possible mechanism for the protective effect of the inhibition of endogenous H_2S synthesis during sepsis.

4.3 Materials and Methods

Animals: Male Sprague-Dawley rats (Charles River Laboratories, Fayetteville, NC) weighing 253 ± 12 grams were used in this study. Rats were housed in a temperature-controlled environment on 12-hour light/dark cycles with free access to standard chow and water. All animal manipulation was in strict adherence with National Institutes of Health guidelines and experimental protocols were approved by Institutional Animal Care and Use Committee of the University of North Carolina at Charlotte.

Reagents: Sodium sulfide (Na_2S), DL-progargylglycine (PAG), fluorescein Isothiocyanate (FITC) on celite, citrate-phosphate-dextrose with adenine (CPDA) and lipopolysaccharide (LPS) (E. Coli, serotype 026:B6) were purchased from Sigma Aldrich (St. Louis, Missouri). Endothelin-1 (ET-1) was purchased from the American Peptide Company (Sunnyvale, CA).

Red Blood Cell Labeling: Red blood cells (RBCs) were fluorescently labeled as described by Zimmerhackl et al [150]. Briefly, 10-12 ml of whole blood was obtained from a donor rat and placed in a tube containing 0.5 ml of heparin (1000 usp units/ml)

(J.A. Webster Veterinary Supply, Devens, MA). Blood was centrifuged at 500g for 10 minutes at 4° Celsius. The plasma supernatant was removed and RBCs were washed 3 times (500g, 10 min, 4° Celsius) with Alsevier's buffer (in g/l: 20.5 glucose, 8.0 citric acid trisodium salt, 0.55 citric acid, and 3.766 NaCl, pH =6.2) and once in bicine buffer (in g/l: 2.264 Bicine, 0.399 NaOH, 7.288 NaCl, pH=8.3). After bicine buffer wash, the RBC pellet was resuspended in a 1:1 ratio (50% hematocrit) with bicine buffer. FITC on celite (4mg/ml of RBC suspension) was dissolved in Dimethylformamide (100 µl/4 mg FITC) and combined with the RBC suspension. After 2 hour incubation with FITC, RBCs were washed once with bicine buffer, followed by 3 saline washes. Following the final wash, RBCs were resuspended to 50% hematocrit ratio with saline. For storage, citrate-phosphate-dextrose with adenine solution (CPDA, 140µl/ml RBC suspension) was added to the RBC suspension. Fluorescently labeled RBCs were kept in the dark at 4° Celsius and used within 7 days of labeling. On the day of the experiment, RBCs were washed once with bicine buffer and 3 times with saline solution. After the final wash, RBCs were resuspended to 50% hematocrit and the RBC suspension (100 µL/100g body weight) was injected into the inferior vena cava.

Intravital Microscopy: Rats were anaesthetized using 1-2% isoflurane (J. A. Webster Veterinary Supply, Devens, MA). A laparotomy was performed to expose the portal vein and associated vasculature. To prevent splenic congestion, blood flow to the spleen was occluded . The splenic vein was isolated and cannulated using a cannula made from PE-50 tubing for measurement of portal venous pressure and infusion of test substances into the portal vein. The right carotid artery was cannulated in order to monitor arterial pressure. Prior to transferring the rat to the microscope, FITC-labeled red

blood cells were injected into the inferior vena cava (100 µL/100g body weight at 50% hematocrit).

For microscopy, rats were placed on an Olympus IX70 inverted microscope (Olympus America, Melville, NY) and situated on a motorized stage with a viewing window over the objective lens. The liver was exteriorized and the left lobe positioned over the viewing window to allow for epi-illumination. The internal organs were covered with saran wrap and saline-soaked gauze to prevent evaporative loss. The splenic cannula was connected to a blood pressure analyzer (Digi-Med low-pressure analyzer; Micro-Med, Louisville, KY) and a syringe pump (Harvard Apparatus, Holliston, MA) via a t-tube fitting to allow for simultaneous monitoring of portal pressure (PP) and infusion of treatment substances. The carotid cannula was connected to a Digi-Med high-pressure analyzer to measure mean arterial pressure (MAP) and heart rate (HR). A MouseOx pulse oximeter (Starr Life Sciences, Pittsburgh, PA) was used to monitor arterial oxygen saturation and heart rate to ensure the stability of the rat during the experiment. Two pericentral and two periportal fields were selected at random and programmed into the motorized stage. The motorized stage gives the advantage of repeated imaging of multiple fields over several time points. After selecting the four fields, rats were allowed to rest for 20 minutes before beginning one of the following experimental protocols.

H_2S Infusion: The H_2S donor Na_2S was used to investigate the effect of H_2S on the hepatic microcirculation. In order to determine the effects of infusion, saline was infused through the splenic cannula at a rate of 100 µL/min for the first 10 minutes. Freshly prepared Na_2S (2 µmoles/kg/min) was then infused for 10 minutes, followed by a 10 minute recovery period of saline. Throughout the experiment, MAP, PP, HR, and O_2

saturation levels were monitored and recorded. The sinusoids were visualized using a FITC filter (excitation 494 nm, emission 518 nm) and 40x objective lens. Micrographs were taken every 10 minutes, with the first set taken at the beginning of the experiment. Micrographs were taken using a Cooke Sensicam digital electron multiplier CCD camera (The Cooke Corporation, Romulus, MI).

Endothelin-1 Infusion: Rats were divided into one of four treatment groups: 1. Control, 2. LPS, 3. PAG, 4. PAG/LPS. For the LPS group, rats received an intraperitoneal injection of LPS (1 mg/kg) 6 hours prior to surgery. Previous reports from our lab indicate that the CSE inhibitor PAG has an effect on the hepatic vasculature within 30 minutes of administration. At this early time point, PAG is not expected to have an effect on the development of the inflammatory response following LPS administration. The PAG group received an intraperitoneal injection of PAG (50 mg/kg) 30 minutes prior to surgery. For animals receiving both treatments, LPS was injected 5.5 hours prior to PAG, which was administered 30 minutes before surgery. After the stabilization period, saline was infused for ten minutes in order to establish baseline readings. ET-1 (125 pmoles/kg/min) was infused for 10 minutes following saline infusion. A 30 minute recovery period was allowed after ET-1 infusion, during which time, saline was infused to prevent dehydration. Throughout the entire experiment, MAP, PP, HR, and O_2 saturation levels were monitored and recorded. Micrographs were taken every ten minutes, beginning after the start of the first saline infusion. For sinusoidal diameter and RBC velocity analysis, digital videos were captured using a FITC filter and a 40x objective lens at a frame rate of 15 frames per second for a total of 60 frames. Off line analysis was performed using PTI imagemaster software.

To assess hepatic oxygen redox potential, lower magnification micrographs (4x objective) were collected to assess global NADH autofluorescence (excitation 345 nm, emission 455 nm) as previously described [99]. NADH is fluorescent at this wavelength while NAD+ is not. An increase in fluorescence intensity correlates with an increase in the NADH/NAD+ ratio. This serves as an indirect assessment of oxygen availability. All camera settings (gain, binning, black level, contrast) were the same for all NADH micrographs.

Off-line image analysis**:** Off-line image analysis was performed using PTI imagemaster software (Photon Technology International Inc., Birmingham, NJ). For sinusoidal diameter and heterogeneity of diameter data, 40x micrographs taken with a FITC filter were used. Four vessels were randomly selected per field. Sinusoidal diameter was determined by calculating the total area of a vessel and dividing it by the length of the vessel (Width = Area/Length). The mean sinusoidal diameter represents the four vessels from four different fields per animal. The standard deviation of individual vessels within one field was used to assess the heterogeneity of perfusion. Vessel data presented is the mean between two different researchers performing independent analysis.

To assess the percentage of sinusoids perfused, 4 vessels per field were randomly chosen. Vessels were considered perfused if blood flow was visible during the 60 micrograph video, irrespective of flow velocity. The mean sinusoidal perfusion for each animal is representative of four vessels from four separate fields.

Red Blood Cell Tracking for Velocity Distribution: We developed an automated method to detect and track red blood cells during video playback of micrograph sequences. To detect red blood cells, a classification model was trained to

learn the appearance of red blood cells [1]. For each frame in a given sequence, red blood cells were detected based on a set of features that detect the visual differences between pixels belonging to cells or the background. These features included mean intensity, standard deviation of intensity and the difference between the mean intensity of pixels closer to the center of patch and the pixels farther away. Using connected component labeling, cell pixels were assigned to cell regions [2]. RBCs were tracked by corresponding cell regions in successive frames using a tracking algorithm as previously described [3]. For each cell, we dynamically predicted its motion and appearance in the next frame. Tracking of cells between frames was based on the best match as determined by our prediction model. The number of red blood cells and their velocities were then determined from the tracking output of the automated tracking algorithm. For each field analyzed, vessels were grouped based upon mean RBC velocities and the distribution of velocity speeds was reported.

Statistical Analysis: All data are presented as means ± standard error of the mean (SEM). Statistical analysis was performed using Sigma Plot 11 software (Systat Software Inc., San Jose, CA). Statistical significance was determined by one or two way ANOVA with independent and repeated measures used where appropriate. Student-Newman-Keuls *post hoc* test was used when statistical differences were detected. Log transformation was performed when data did not pass normality and equal variance tests. Statistical significance was set at $P<0.05$.

4.4 Results

Effect of portal Infusion of H_2S on hepatic hemodynamics: We investigated the effect of portal infusion of the H_2S donor Na_2S (2 μmoles/min/kg) on hepatic hemodynamics *in vivo*. H_2S infusion increased portal pressure to 8.6 ± 0.8 mmHg from 6.8 ±0.2 mmHg during baseline saline infusion (Figure 27, $P<0.05$). After H_2S infusion was stopped, portal pressure returned to baseline within 10 minutes (6.9 ± 0.2 mmHg). Mean arterial pressure (MAP) and heart rate (HR) were monitored to determine the effect of H_2S infusion on systemic hemodynamics. There was a slight drop in heart rate during infusion of H_2S (317 ± 11.4 baseline vs. 305 ± 11.7 BPM during H_2S infusion, Table 1) which persisted through the recovery (302 ± 11.1 BPM). Although this decrease was statistically significant ($P<0.05$), it is not likely to be functionally important. Infusion of H_2S did not affect MAP when compared to controls (100 ± 4.0 mmHg vs. 102 ± 2.8 mmHg, Table 1, P=NS); however, there was a small overall drop in MAP at the end of the recovery period (96.7 ± 4.6 mmHg, $P<0.05$) which is likely an effect of the surgical procedure.

Because H_2S was acting locally on the hepatic hemodynamics, we used intravital microscopy to determine if H_2S infusion has an effect on the hepatic sinusoids. H_2S infusion produced a net decrease in sinusoidal diameter (5.7 ± 0.3 μm after H_2S vs. 6.2 ± 0.3 μm at baseline, Figure 28, $P<0.05$). 10 minutes after H_2S infusion was ceased, sinusoidal diameter had nearly returned to baseline (6.0 ± 0.4 μm). Sinusoids adjacent to constricted sinusoids have been shown to be dilated following inflammatory stress. Therefore, we compared the variability between sinusoidal diameters within each field analyzed. One way ANOVA analysis demonstrated a significant effect of treatment over

the course of the experiment (Figure 29, $P<0.05$) suggesting an increase in sinusoidal diameter heterogeneity following H_2S infusion.

Effect of PAG treatment on the hepatic microcirculation during endotoxemia: Our previous results demonstrated a modest constrictor effect of exogenous H_2S on the hepatic sinusoid. Since hyperconstriction of the sinusoid is a contributing factor to hepatic injury during endotoxemia, we used the non-competitive inhibitor of CSE, dl-propargylglycine (PAG), to investigate the contribution of endogenous H_2S to hepatic sinusoidal sensitization to the constrictor effect of ET-1 during endotoxemia. A total of twenty rats were divided evenly into four groups; control, LPS (1mg/kg ip, 6 hr), PAG (50 mg/kg ip, 30 minutes before microscopy), and cotreatment with LPS/PAG. Systemic hemodynamics (MAP and HR) were monitored throughout the experiment and no biologically significant changes were observed (Table 2). There was no significant difference in baseline portal pressure among the treatment groups. 10 minute infusion of ET-1 (125 pmoles/kg/min) resulted in an increase in portal pressure in control rats (121% of baseline pressure, Figure 30, $P<0.05$). While PAG treatment alone had no effect on the ET-1 response, the constrictive effect of ET-1 was exacerbated in endotoxemic rats (175% of baseline, $P<0.01$). PAG treatment significantly attenuated the increased constrictor effect of ET-1 in endotoxemic animals (143% ± 6.9, $P<0.05$ compared to LPS alone).

Using intravital microscopy, we demonstrated that infusion of ET-1 reduced sinusoidal diameter in control and PAG treated rats by 12.6% and 12.3%, respectively (Figure 31). In LPS treated rats, the effect of ET-1 was greatly potentiated (30.9 % reduction in diameter, $P<0.01$ vs. control) similar to our previous reports [12]. Treatment

with PAG abrogated the sensitization of the sinusoid to the constrictor effect of ET-1 resulting in a decrease in sinusoidal diameter similar to controls (11.6 % reduction). Since we previously reported the presence of constricted and dilated sinusoids following inflammatory stress, we assessed the variability of sinusoidal diameters among treatment groups. LPS produced a significant increase in the standard deviation of the means of sinusoidal diameters per field (1.9 ± 0.5 μm vs. 0.9 ± 0.2 μm in controls, Figure 32, $P<0.05$) which was attenuated by PAG treatment (0.8 ± 0.1 μm, $P<0.05$). Interestingly, PAG alone produced a significant increase in heterogeneity of sinusoidal diameters during ET-1 infusion (1.5 ± 0.3 μm, $P<0.05$ vs. control).

There was a clear difference between treatment groups in sinusoidal diameter and heterogeneity based on the visual appearance of the sinusoids (Figure 33). We wanted to investigate if these physical differences between groups produced functionally important alterations in sinusoidal perfusion. To do this, FITC-labeled RBCs were tracked as they traveled through individual sinusoids using a computer tracking algorithm we developed. For each group, we calculated the mean RBC velocity through individual sinusoids. There was no difference between treatment groups prior to ET-1 infusion. While there was no statistical difference in the mean velocities of RBC between groups following ET-1, the distribution of mean sinusoidal RBC velocities was different (Figure 34). The distribution of sinusoids with a given RBC velocity followed a standard Gaussian distribution in control animals after ET-1 infusion. In LPS treated rats there were a greater number of occluded and slow flowing sinusoids distorting the Gaussian distribution. PAG treatment restored the distribution of sinusoidal velocities in LPS treated rats making it more similar to control animals.

The presence of several completely occluded sinusoids following ET-1 infusion prompted us to determine the sinusoidal perfusion percentage before and after ET-1 infusion in each group (Figure 35). There was no difference between the groups in the percentage of sinusoids perfused (all groups > 90%) prior to infusion of ET-1. ET-1 infusion caused a decrease in sinusoidal perfusion percentage among all treatment groups (Figure 36). In the LPS group, only 63% of the sinusoids were perfused following ET-1 infusion, which was significantly less than the control group (91% perfused, $P<0.001$). In LPS treated animals, PAG treatment significantly improved sinusoidal perfusion (78% perfused, $P<0.05$) after ET-1 treatment.

Based on these results, we investigated whether PAG treatment increased oxygen delivery to the liver during ET-1 infusion. Using NADH autofluorescence as an indicator of cellular redox potential allows for indirect assessment of oxygen availability. NADH fluorescence remained unchanged following infusion of ET-1 in control and PAG rats (Figure 36). LPS treatment was associated with a significant increase in NADH autofluorescence (61 grey scale units vs. 9.6 units in control, Figure 37, $P<0.05$). PAG treatment attenuated the increase in NADH fluorescence following ET-1 infusion in endotoxemic rats (21 grey scale unit increase, $P<0.05$ vs. LPS).

4.5 Discussion

The present study investigated whether hydrogen sulfide (H_2S) modulates sinusoidal perfusion within the hepatic microcirculation. We show that portal infusion of the H_2S donor Na_2S *in vivo* causes a modest increase in hepatic resistance which is due in part to sinusoidal constriction. Furthermore, we demonstrate that treatment with PAG attenuates the hypersensitization of the hepatic sinusoid to the vasoconstrictive effect of

ET-1during endotoxemia. Taken together, our results show that H_2S affects sinusoidal perfusion in a manner distinct from its action in the portal venules and it significantly contributes to microvascular dysregulation in endotoxemia.

Historically consisting of nitric oxide (NO) and carbon (CO), the "gasotransmitters" are a group of endogenously synthesized gases that act as mediators in a multitude of physiological and pathophysiological functions [135]. The newest recognized member of this group, H_2S, is primarily produced during cysteine metabolism by two pyridoxal 5' phosphate dependent enzymes, cystathionine β synthase (CBS) and cystathionine γ lyase (CSE) with the latter being the major source of H_2S in the liver and cardiovascular system [18, 48, 123]. Like NO and CO, H_2S participates in the regulation of the blood flow by modulating vascular resistance. Evidence for H_2S-induced vasodilation has been provided in several different experimental models, including isolated aortic rings and mesenteric artery beds [21, 48]. Whereas NO and CO mediated relaxation of VSMCs is cGMP dependent [39, 51], H_2S increases the activity of K_{ATP} channel activity in VSMCs leading to hyperpolarization and relaxation [148]. A common criticism regarding *in vitro* studies with H_2S is the large amount of exogenous H_2S required to induce vasodilation which may not reflect local concentrations *in vivo*. However, $CSE^{-/-}$ mice spontaneously develop hypertension which provides evidence for the important contribution of endogenously produced H_2S in vasoregulation [140].

Despite the large body of evidence that demonstrates the vasodilatory action of H_2S, there are reports that H_2S can produce vasoconstriction in certain circumstances. At low concentrations, the H_2S donor NaHS reverses the relaxation of aortic rings to acetylcholine [74]. Acetylcholine vasodilation is NO dependent. Since H_2S had only a

minor effect in the absence of acetylcholine, it is likely that H_2S-induced vasoconstriction results from an interaction with NO. This study also suggested an NO independent mechanism of vasoconstriction that involves reduction of cAMP levels in the presence of NaHS [74]. *In vivo* studies demonstrate that intravenous infusion of H_2S produces a small, transient increase in blood pressure which is abrogated in mice treated with L-NAME, an inhibitor of nitric oxide synthase (NOS) [4]. Thus, the interaction between NO and H_2S is likely complex. H_2S can directly inhibit all three isoforms of recombinant NOS [65-66]; however, reports also show an increase in eNOS activity from H_2S treatment in bovine arterial endothelial cells (BAECs) [102]. In addition to an effect on NOS, H_2S may interact with NO to form a vaso-inactive nitrosothiol, quenching the NO signal [137]. Due to the ambiguous vasoactive role of H_2S in vasoregulation, we first investigated the effect of portal infusion of H_2S on hepatic vascular resistance *in vivo*. Infusion of the H_2S donor Na_2S produced a modest increase in portal pressure. Previously, we demonstrated that the liver has a high capacity to metabolize H_2S [89]. Since we infused H_2S directly into the portal circulation via the splenic vein, it is expected that H_2S would have no effect on systemic hemodynamics as all should be metabolized in a single pass through the liver. Fluctuations in portal pressure result from alterations in either splanchnic blood flow or a change in intrahepatic resistance. Since portal infusion of H_2S should have no effect on splanchnic blood flow, the change in portal pressure is most likely due to an increase in intrahepatic resistance. Using intravital microscopy, we observed a reduction in sinusoidal diameter during infusion of H_2S. Kamoun et al reported that ET-1 stimulated sinusoidal constriction produces heterogeneous blood flow at the level of individual sinusoids [57]. In our study, H_2S

infusion increased the heterogeneity of sinusoidal diameters indicative of increased heterogeneity of flow. These findings imply that the increase in portal pressure observed during infusion of H_2S is the result of increased intrahepatic resistance resulting from modest net sinusoidal constriction.

The finding that H_2S causes constriction of the sinusoids suggests a fundamentally different mechanism of vascular tone regulation in the sinusoid compared to other resistance vessels. H_2S hyperpolarizes vascular smooth muscles cells (VSMC) via activation of the K_{ATP} channel which leads to relaxation and vasodilation [128]. Importantly, H_2S-induced vasodilation is reversible with treatment of the K_{ATP} channel inhibitor, glibenclamide [148]. Within the liver, VSMCs are present around the portal terminal venules and hepatic arterioles. Accordingly, studies have shown that H_2S attenuates norepinephrine (NE) induced vasoconstriction in isolated livers and increases the hepatic arterial buffer response (HABF) [33, 115]. However, these findings suggest that H_2S acts as a vasodilator in the liver at sites where VSMCs are present. The hepatic sinusoids lack VSMCs. Rather, sinusoidal tone is primarily regulated by hepatic stellate cells (HSCs). These specialized pericytes surround individual sinusoids and respond to local balances between vasodilators and vasoconstrictors [62]. An imbalance between vasoactive molecules can cause HSC contraction or relaxation and alter sinusoidal tone. During sepsis, an imbalance between NO and the vasoconstrictive effect of ET-1 produces sinusoidal hyperconstriction [12]. In an isolated, perfused liver, Fiorucci et al demonstrated that H_2S attenuated catecholamine-induced vasoconstriction and may be beneficial during cirrhosis by reducing portal hypertension and increasing hepatic perfusion [33]. One would predict that the vasodilatory effect of H_2S would also be

beneficial during sepsis; however, we recently reported that H_2S differentially modulates the effect of PE and ET-1 in an isolated, perfused liver. It is possible that H_2S may have a different effect on the hepatic sinusoids than on the portal venules. Since sinusoidal hyperconstriction and heterogeneous perfusion contribute to liver injury during sepsis [12-13], we hypothesized that the inhibition of the H_2S-producing enzyme, CSE, would attenuate hepatic sinusoidal dysfunction in an endotoxin model of sepsis.

In the sinusoid, the vasoconstrictive effect of ET-1 results from an interaction with ET_A and ET_B receptors expressed in hepatic stellate cells (HSCs) [49]. Under normal conditions, this vasoconstrictive effect is counteracted by ET-1 interaction with ET_B receptors in sinusoidal endothelial cells (SECs) which increases eNOS activity [98]. ET-1 levels increase following inflammatory stress [3, 109]; however, more importantly ET-1 stimulated NO production from SECs is impaired, in part, due to an increase in caveolin 1 which has been shown to inhibit eNOS activity [67]. This leads to sinusoidal hypersensitization to the vasoconstrictive effect of ET-1. A consequence of sinusoidal constriction is the development of heterogeneous sinusoidal flow and focal hypoxia [57]. Individual constricted sinusoids are under-perfused, resulting in local ischemia, while neighboring sinusoids are dilated and over-perfused. While total hepatic blood flow may remain constant, the heterogeneous blood flow fails to match O_2 supply with demand, producing hepatic dysfunction and injury.

For this study, we used a mild inflammatory stress (LPS 1mg/kg ip, 6 hours) to assess the effect of PAG treatment on the early changes in hepatic microcirculation during endotoxemia. There was no effect on systemic hemodynamics at this dose and time point (Table 2). Therefore, the results represent a local hepatic effect rather than a

consequence of systemic alterations. Despite our prior result that demonstrated an effect of intraportal infusion of H_2S on the hepatic sinusoid, PAG treatment did not alter the hepatic microcirculation before or after ET-1 infusion in control animals. Since the liver has a high capacity to metabolize H_2S, it is likely that endogenous H_2S levels are prevented from accumulating to levels sufficient to exert a vasoactive function under normal conditions. However, there is an increase in hepatic CSE expression and H_2S synthesis during sepsis [144]. Therefore, it is possible that elevated H_2S levels are sufficient to contribute to sinusoidal dysfunction in endotoxemic rats.

A study by Zhang et al demonstrated that ET-1 causes sinusoidal constriction which colocalizes with HSCs [146]. Kamoun and coworkers observed that portal infusion of ET-1 increased the heterogeneity of perfusion through the sinusoids [57]. Several studies demonstrated that inflammatory stress, including sepsis, enhances the vasoconstrictive effect of ET-1 in the hepatic microcirculation [12, 58, 94]. Furthermore, Baveja et al demonstrated that sinusoidal hyperconstriction and heterogeneous blood flow lead to impaired O_2 delivery to hepatic cells resulting in tissue hypoxia and injury [13]. In the present study, we demonstrate that endotoxin significantly increases sinusoidal constriction and heterogeneity of sinusoidal perfusion. Additionally, there were more non-perfused sinusoids in LPS treated rats following ET-1 infusion when compared to control. These effects were reversed by a single injection of PAG 30 minutes prior to experimental treatments. In endotoxin treated animals, PAG treatment attenuated sinusoidal hyperconstriction and decreased perfusion heterogeneity following ET-1 infusion. PAG treatment also reduced the number of non-perfused sinusoids after ET-1 infusion.

Since PAG treatment significantly improved microcirculatory function after ET-1 infusion in LPS treated rats, we investigated if it also improved O_2 delivery to the liver tissue. Using intravital microscopy, we can exploit the fact that the reduced form of the cellular electron carrier, nicotinamide adenine dinucleotide (NADH), is fluorescent when excited with UV light, while the oxidized form, NAD^+ is not. Because O_2 is the final electron acceptor in the electron transport chain, a deficiency in O_2 will lead to an increase in $NADH/NAD^+$. Previous work from our lab demonstrated that hepatic NADH autofluorescence is highly correlated to tissue PO_2 [99]. Therefore, we can interpret an increase in fluorescence as a decrease in hepatic O_2 availability. In the present study, infusion of ET-1 caused a significant increase in NADH fluorescence in animals treated with endotoxin. ET-1 infusion increased NADH fluorescence in all animals with the greatest increase occurring in LPS treated rats. In agreement with our sinusoidal parameters, PAG treatment reduced the increase in NADH fluorescence in LPS treated animals suggesting improved O_2 delivery to the hepatic tissue.

While NADH is an indirect assessment of hepatic O_2 availability, our finding is in agreement with previous reports from our lab. Using an isolated, perfused liver system, we demonstrated that infusion of low concentrations of ET-1 increased O_2 consumption in control livers. In LPS treated animals, O_2 consumption was inhibited by infusion of low concentration of ET-1 due to an increase in vascular resistance (presumably sinusoidal) and impaired O_2 delivery. In endotoxemic livers, PAG treatment restored O_2 consumption following low dose ET-1 treatment. Taken together, our findings suggest that PAG maintains O_2 delivery to the liver during endotoxemia.

Our results demonstrate one possible protective mechanism of CSE inhibition during sepsis. It is possible that a portion of the protective effect of PAG occurs independently of H_2S. However, several other studies show that the protective effect of PAG is reversible with treatment with the rapid releasing H_2S donor NaHS [5, 143, 145]. Furthermore, H_2S likely does not circulate in the blood; rather, endogenous H_2S levels are the summation of continuous local production and disposal [92]. Therefore, we focused on inhibiting endogenous H_2S synthesis to study the effect of H_2S during endotoxemia because it is more likely to be physiologically relevant than the high concentrations of H_2S needed to observe *in vivo* effects.

Importantly, several studies show a protective effect of PAG pre-treatment on the inflammatory response during sepsis [143]. We specifically chose to treat with PAG for 30 minutes immediately before all microscopy or liver isolation surgery. By administering PAG five and a half hours after LPS treatment, we likely mitigate the difference in the inflammatory response between the LPS and PAG/LPS groups. Therefore, we can interpret our results as the acute effect of the presence of H_2S in the hepatic sinusoid and not as the consequence of an altered inflammatory response.

There are conflicting reports of the vasoregulatory actions of H_2S in the microcirculation. H_2S likely provides a beneficial effect during fibrosis and cirrhosis. In these chronic conditions, H_2S, acting as a vasodilator in the portal venules, likely improves hepatic perfusion and reduces portal hypertension. However, during sepsis, the failure of the hepatic microcirculation occurs primarily at the level of the sinusoids which are hyperconstricted. For the first time, we directly demonstrate an effect of H_2S in the regulation of sinusoidal tone. The vasoconstrictive effect of H_2S in the sinusoids is

fundamentally different than the previously reported dilatory effect of H_2S in presinusoidal resistance vessels. Furthermore, because endotoxemia is associated with hepatic sinusoidal hyperconstriction, we investigated if inhibition of CSE significantly increases hepatic microcirculatory dysfunction following LPS treatment. We show that treatment with the CSE inhibitor, PAG, significantly improves hepatic microcirculatory function following ET-1 infusion during endotoxemia. Our results imply that the protective effect of PAG observed during sepsis may be due in part to improved hepatic perfusion. In summary, our results suggest that the current understanding of the role of H_2S in the hepatic microcirculation is incomplete. Rather, a more complex role is likely where H_2S acts as a vasodilatory in the presinusoidal resistance vessels and exerts a constrictor effect in the hepatic sinusoids which may contribute to hepatic microcirculatory dysfunction during sepsis.

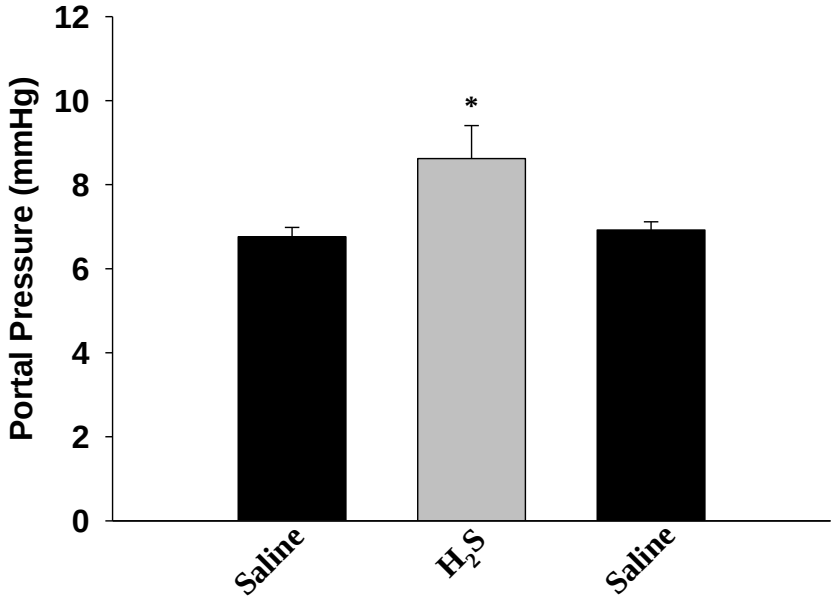

Figure 27: Effect of portal infusion of Na_2S on portal pressure *in vivo*. Portal pressure was monitored to determine the effect of the H_2S donor Na_2S on intrahepatic resistance. The peak portal pressure during 10 minute infusion Na_2S was recorded and compared to baseline and 10 minutes following Na_2S infusion. Data are presented as the mean ± SEM from 5 separate experiments. Statistical analysis was performed using 1 way repeated measures ANOVA. * = $P<0.05$.

Table 1: Effect of portal infusion of Na_2S on mean arterial pressure (MAP) and heart rate (HR)**.** MAP and HR were monitored to determine the systemic effect of portal infusion of Na_2S. Data are presented as the mean ± SEM from 5 separate experiments. Statistical analysis was performed using 1 way repeated measures ANOVA. * = $P<0.05$.

	Saline	H_2S	Saline
Mean Arterial Pressure (mmHg)	102 ± 2.9	99.9 ± 4.0	96.7 ± 4.6 *
Heart Rate (BPM)	317 ± 11.4	305 ± 11.6 *	301 ± 11.1 *

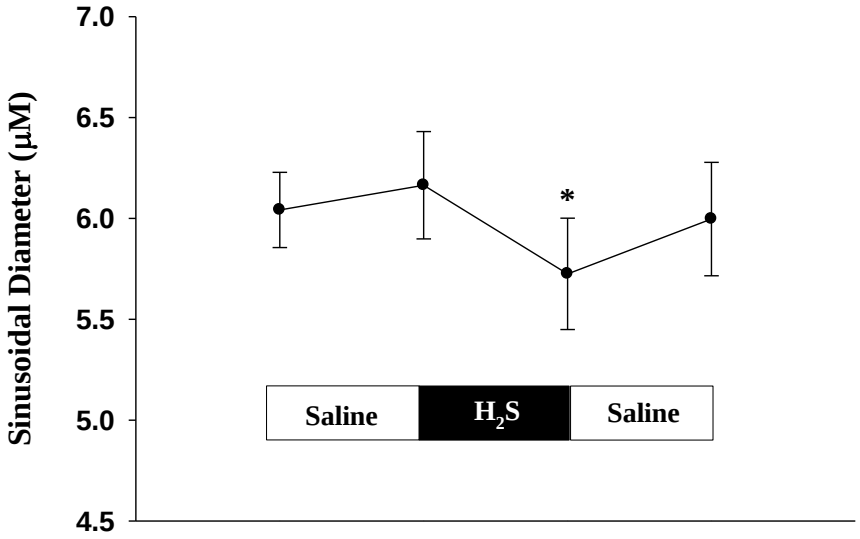

Figure 28: Effect of Na_2S on sinusoidal diameter**.** *In vivo* micrographs of the hepatic sinusoids were taken before, during, and 10 minutes following infusion of H_2S donor Na_2S. Infusion of Na_2S decreased sinusoidal diameter. Data are presented as the mean ± SEM from 5 separate experiments. Statistical analysis was performed using 1 way repeated measures ANOVA. * = $P<0.05$.

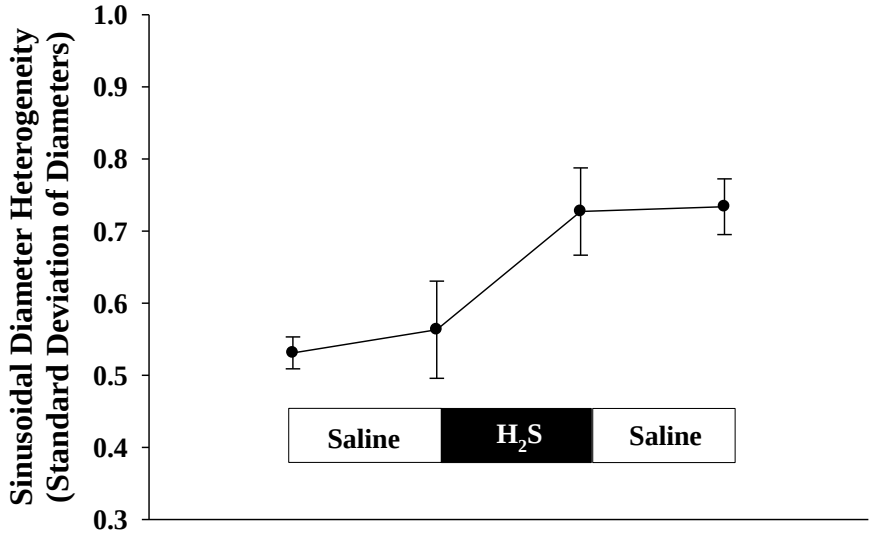

Figure 29: Effect of Na_2S on heterogeneity of sinusoidal diameters. *In vivo* micrographs of the hepatic sinusoids were taken before, during, and 10 minutes following infusion of H_2S donor Na_2S. Infusion of Na_2S decreased sinusoidal diameter. Data are presented as the mean ± SEM from 5 separate experiments. Statistical analysis was performed using 1 way repeated measures ANOVA and demonstrated a significant overall effect of treatment ($P<0.05$).

Table 2: Effect of portal infusion of ET-1 on mean arterial pressure (MAP) and heart rate (HR). MAP and HR were monitored to determine the systemic effect of portal infusion of ET-1 within treatment groups. Data are presented as the mean ± SEM from 5 separate experiments. Statistical analysis was performed using 2 way ANOVA. * = P<0.05 compared to baseline within treatment group, # = P<0.05 between treatment groups.

				Recovery		
Treatment		Saline	ET-1	10 min	20 min	30 min
Control	Mean Arterial Pressure (mmHg)	127.1 ± 7.2	124.3 ± 10.3	132.3 ± 6.9	130.2 ± 6.5	123.9.2 ± 6.9
	Heart Rate (BPM)	400.0 ± 9.3	393.5 ± 15.1	289.1 ± 16.0	392.1 ± 14.7	389.0 ± 12.5
PAG	Mean Arterial Pressure (mmHg)	135.7 ± 2.6	136.5 ± 2.8	143.0 ± 6.3 *	142.5 ± 2.3	135.0 ± 6.6
	Heart Rate (BPM)	394.3 ± 16.0	396.7 ± 13.4	379.2 ± 19.0	363.6 ± 12.1 #	388.3 ± 19.5
LPS	Mean Arterial Pressure (mmHg)	131.3 ± 4.3	129.3 ± 4.8	133.2 ± 1.8	132.4 ± 8.4	124.4 ± 8.4
	Heart Rate (BPM)	434.7 ± 19.8	432.4 ± 15.0	416.9 ± 14.9	435.1 ± 115.8	398.3 ± 15.8
PAG/LPS	Mean Arterial Pressure (mmHg)	126.6 ± 5.5	129.2 ± 7.4	138.4 ± 9.0 *	134.6 ± 7.4	133.3 ± 8.7
	Heart Rate (BPM)	387.8 ± 19.0	391.2 ± 18.6	384.4 ± 20.0	362.8 ± 21.7 #	401.9 ± 6.2

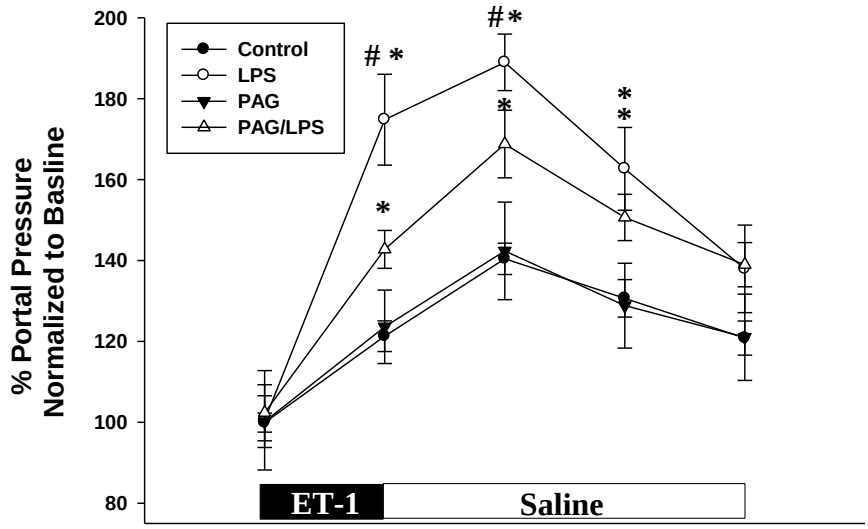

Figure 30: Effect of dl-propargylglycine (PAG) on portal pressure during infusion of endothelin-1 (ET-1) during endotoxemia. Rats were treated with LPS for 6 hours with or without PAG treatment 30 minutes before microscopy. Portal pressure was monitored during infusion of ET-1. The percent increase over baseline is plotted as the mean ± SEM (N=5). Statistical analysis was performed using two way ANOVA with SNK *post hoc* test. * = $P<0.05$ compared to baseline, # = $P<0.05$ between LPS and PAG/LPS.

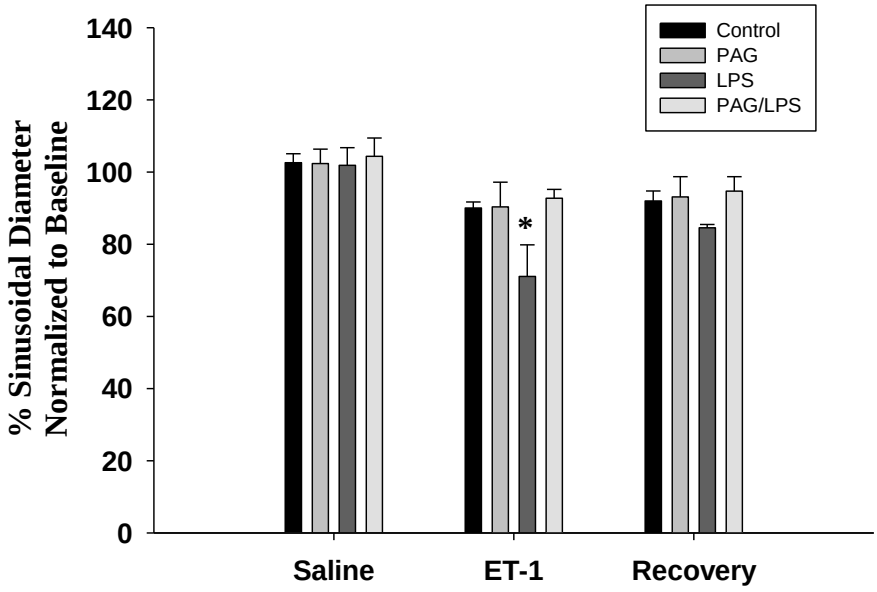

Figure 31**:** Effect of dl-propargylglycine (PAG) on mean sinusoidal diameter during infusion of ET-1 during endotoxemia. Rats were treated with LPS for 6 hours with or without PAG treatment 30 minutes before microscopy. Micrographs of sinusoids were taken before, during, and 10 minutes after ET-1 infusion. PAG attenuates sinusoidal hyperconstriction following ET-1 infusion during endotoxemia. Data are presented as the mean ± SEM from 5 separate experiments. Statistical analysis was performed using 2 way ANOVA with SNK *post hoc* test. * = $P<0.05$.

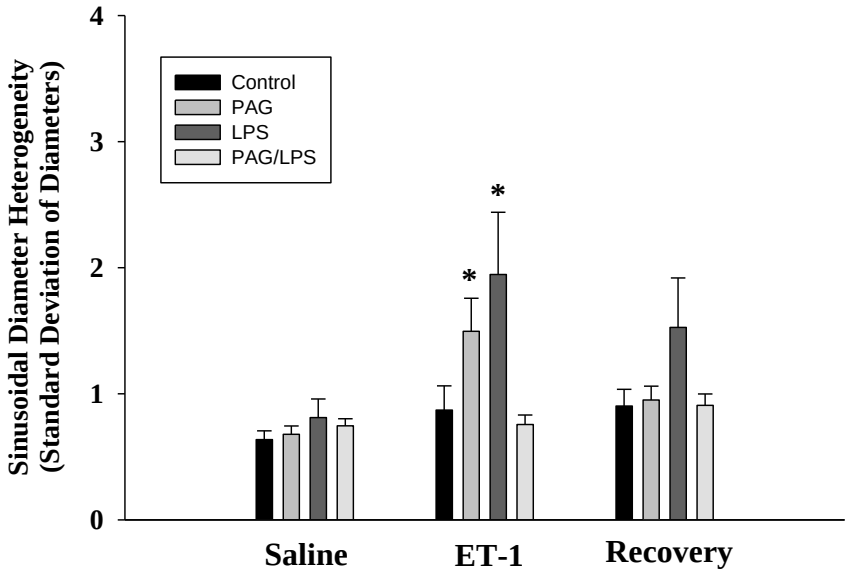

Figure 32: Effect of dl-propargylglycine (PAG) on sinusoidal diameter heterogeneity during infusion of ET-1 during endotoxemia. Rats were treated with LPS for 6 hours with or without PAG treatment 30 minutes before microscopy. Micrographs of sinusoids were taken before, during, and 10 minutes after ET-1 infusion. PAG reduces the heterogeneity of sinusoidal diameter means. Data are presented as the mean ± SEM from 5 separate experiments. Statistical analysis was performed using 2 way ANOVA with SNK *post hoc* test. * = $P<0.05$.

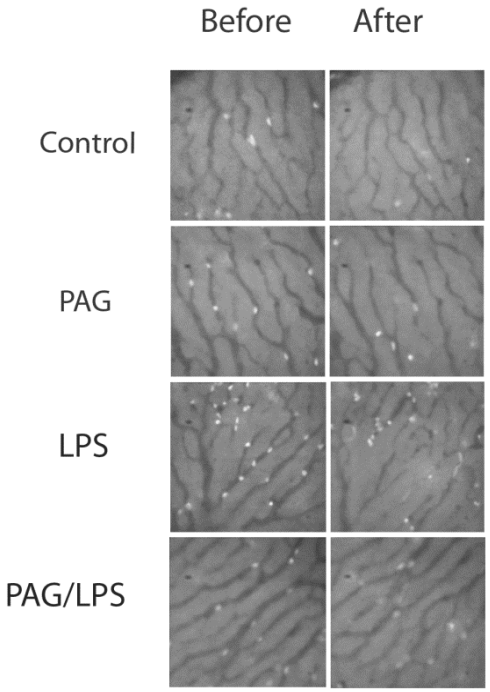

Figure 33: The effect of ET-1 infusion on the hepatic sinusoids following PAG treatment in endotoxemia. Sinusoids were visualized to determine the effect of ET-1 infusion on LPS-treated rats with or without the CSE inhibitor, dl-propargylglycine (PAG). Representative micrographs are shown before and after infusion of 125 pmoles/kg/min infusion of ET-1.

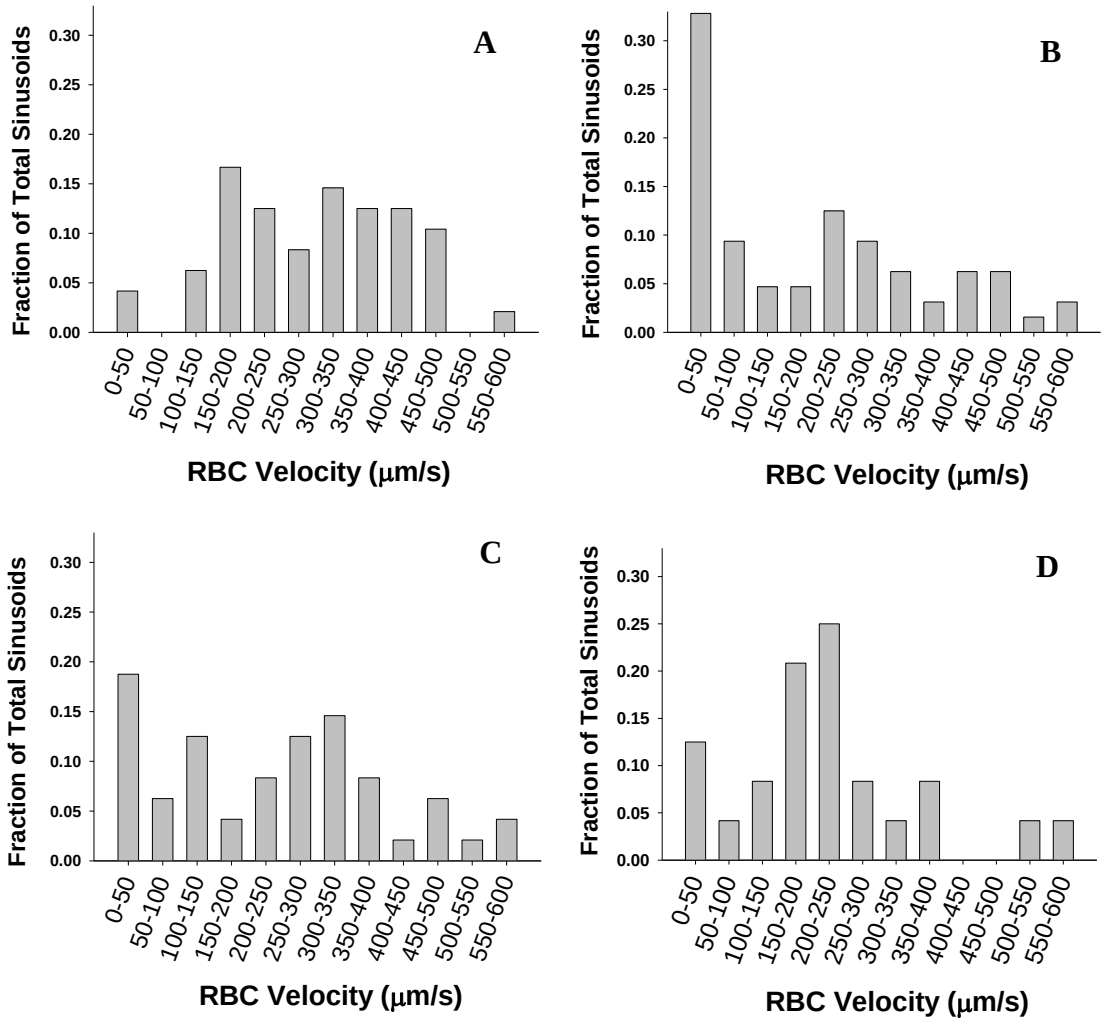

Figure 34: Distribution of sinusoidal flow velocities**.** The distribution of flow velocities through individual sinusoids was determined by tracking FITC –labeled RBCs moving through the sinusoids. The distribution of sinusoidal velocities is plotted as the fraction of total sinusoids within a given velocity range. **A.)** Control **B.)** LPS **C.)** PAG **D.)** PAG/LPS.

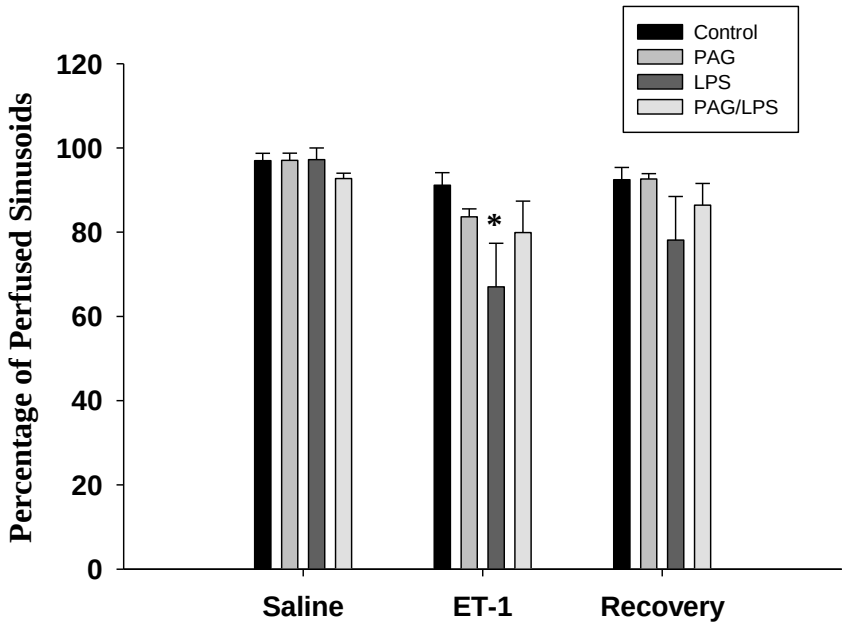

Figure 35: Effect of PAG treatment on sinusoidal perfusion percentage following ET-1 infusion during endotoxemia. The percentage of perfused sinusoids was determined in all treatment group. Data are presented as the mean ± SEM from 5 separate experiments. Statistical analysis was performed using 2 way ANOVA with SNK *post hoc* test.
* = $P<0.05$.

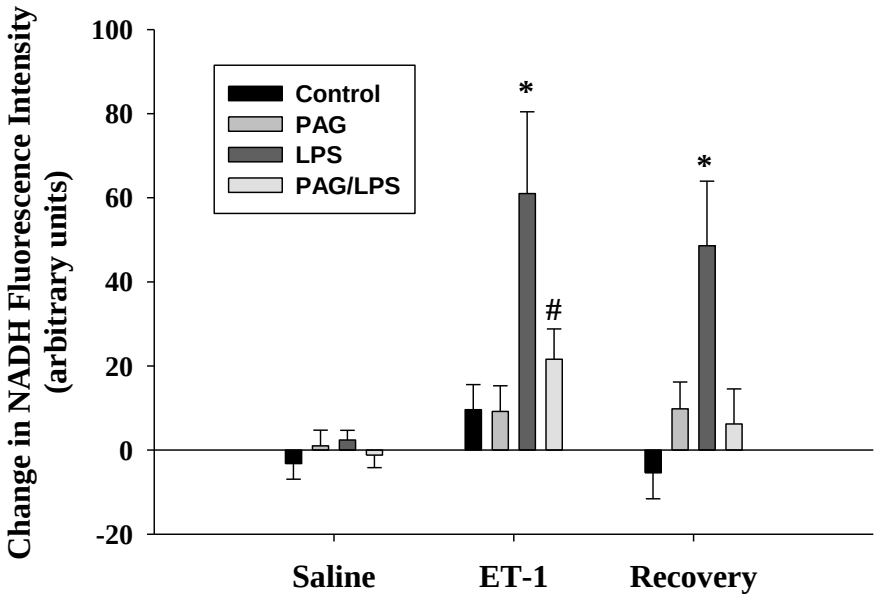

Figure 36: Effect of PAG treatment on hepatic redox potential following ET-1 infusion during endotoxemia. NADH autofluorescence was used as an indirect assessment of the effect of PAG on hepatic O_2 delivery during endotoxemia. An increase in fluorescence is correlated to an increase in NADH/NAD^+ and indirectly a decrease in hepatic O_2 content. Data are presented as the mean ± SEM from 5 separate experiments. Statistical analysis was performed using 2 way ANOVA with SNK *post hoc* test.
* = $P<0.05$, # = $P<0.05$ between LPS and PAG/LPS.

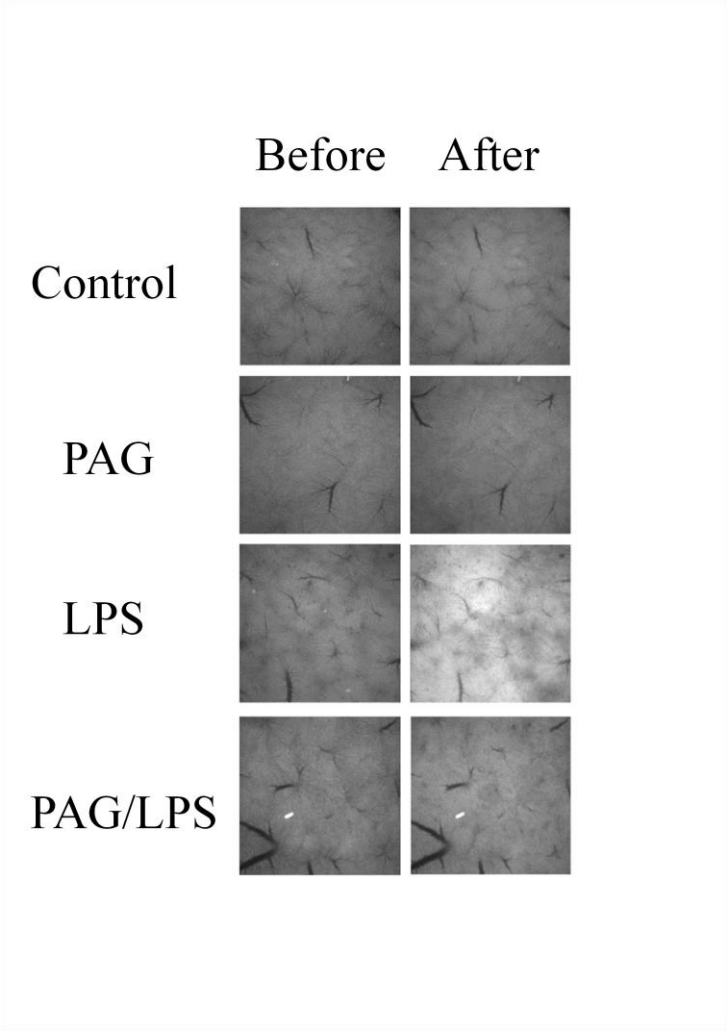

Figure 37: Representative micrographs of NADH autofluorescence before and after ET-1 infusion during endotoxemia. NADH autofluorescence is used as an indirect assessment of hepatic O_2 availability. An increase in fluorescence is associated with an increase in the NADH/NAD^+ ratio and indirectly a decrease in hepatic O_2 availability.

CHAPTER 5: DISCUSSION

The role of hydrogen sulfide (H_2S) in inflammation and disease is poorly understood. Somewhat paradoxically, H_2S is cytoprotective following hepatic ischemia/reperfusion injury, but contributes to disease progression during sepsis [54, 144]. It is well established that hepatic dysfunction is a major early complication in sepsis [38]. Our lab previously focused on the major contribution of the gasotransmitter, nitric oxide (NO), in hepatic dysfunction during sepsis [25]. It is evident that NO has a primary role in sepsis; however, the complex septic milieu in the hepatic microenvironment suggests a more dynamic pathophysiology. Several studies have reported similarities between NO and H_2S in the cardiovascular system [135]. While the importance of NO in the liver vasculature is well documented, few have investigated the effects of H_2S in the hepatic microcirculation, particularly following inflammatory stress. Therefore, this dissertation focused on the effect of H_2S in the liver during sepsis with particular emphasis on hepatic O_2 availability and microcirculatory dysfunction.

A combination of endogenous and exogenous H_2S results in elevated hepatic sulfide exposure during sepsis. Endogenously, cystathionine λ lyase (CSE) is the primary source of H_2S in the liver [56, 147]. Following endotoxin treatment or cecal ligation and puncture (CLP), hepatic CSE levels increase which results in a greater hepatic capacity to synthesize H_2S [72, 144]. As the major recipient of gastrointestinal blood flow, the liver is exposed to an exogenous source of H_2S derived from sulfate-reducing bacteria in the

gut [15]. During septic peritonitis, it is likely that increased production of H_2S from intestinal bacteria may enter the portal circulation and increase hepatic sulfide exposure; however, the fate of elevated H_2S levels in the liver is unclear.

In the presence of toxic levels of H_2S, colonic mucosal cells rapidly detoxify H_2S generated by colonic bacteria, thereby preventing accumulation [37]. Located in the mitochondria, the sulfide quinone reductase (SQR) protein complex couples the oxidation of H_2S with complex Q of the electron transport chain [68]. The oxidation of H_2S by the SQR consumes molecular oxygen at a ratio of 1.5 moles of O_2 per 1 mole of H_2S. In isolated mitochondria from chicken livers, H_2S oxidation was associated with increased O_2 consumption and was coupled to ATP synthesis [141]. The relationship between H_2S oxidation and O_2 consumption has also been observed in a recirculating isolated and perfused liver [8]. A significant complication during sepsis is the loss of hepatic oxidative catabolism of toxic molecules [2, 64]. Whether sepsis affects hepatic sulfide oxidation is unknown. Since a diminished capacity to oxidize H_2S would be detrimental during the high sulfide levels occurring during sepsis, we hypothesized that the liver retains a high capacity to metabolize H_2S during sepsis in order to prevent toxic hepatic accumulation and to remove H_2S from the circulation.

In a non-recirculating isolated and perfused liver, we demonstrate a biphasic effect of H_2S in the liver, which was not present in an isolated and perfused heart. The liver has a high capacity to metabolize H_2S until a critical level. Above this threshold, toxic amounts of H_2S inhibit the oxidation of H_2S. H_2S clearance from the perfusate was associated with an increase in hepatic O_2 consumption. When the PO_2 of the perfusate was reduced, the oxidation of H_2S decreased proportionally, suggesting that available O_2

is obligatory for H_2S clearance. Infusion of H_2S did not alter total intrahepatic resistance indicating that the changes in O_2 consumption were not due to a vascular effect.

In vivo, erythrocytes may participate in the sequestration of H_2S via an interaction with hemoglobin [43]. It is possible that the lack of RBCs in our experimental settings does not reflect hepatic exposure to H_2S *in vivo*. To investigate this possibility, we used *in vivo* microscopy to monitor hepatic oxygen availability during portal infusion of H_2S. We demonstrated that portal exposure to H_2S is associated with a significant decrease in hepatic O_2 content and redox potential which suggests that elevated H_2S in the portal circulation is cleared by the liver. Interestingly, in preliminary studies, we were unable to detect H_2S immediately after it was added to blood. When the same experiment was performed with plasma, H_2S was detectable, suggesting that RBCs can sequester H_2S. Nevertheless, our findings clearly demonstrate that H_2S in the circulation is available for hepatic oxidation which can decrease hepatic O_2 availability.

Early cardiovascular dysfunction during sepsis is characterized by a hyper-dynamic state. An increase in cardiac output and modest peripheral vasodilation leads to an increase in global DO_2 [44]. Despite this increase in DO_2, it is well established that liver dysfunction during sepsis is due in part to hypoxic injury [12, 53, 100]. The findings from the first part of this dissertation suggest that H_2S may act as a double edged sword during sepsis and contributes to hepatic dysfunction resulting from focal hypoxia. The presence of elevated hepatic H_2S levels would require greater H_2S oxidation to prevent the toxic effects. As a consequence, H_2S oxidation consumes O_2, which would exacerbate hypoxic stress. The inverse relationship between O_2 and H_2S oxidation may partially be responsible for the increase in hypoxia inducible factor (HIF-1α) during endotoxemia

[110]. Indeed, *C. Elegans* grown in H_2S containing medium exhibit elevated levels of HIF-1α nuclear accumulation [17]. Since HIF-1α enhances the pro-inflammatory response during endotoxemia [75, 100], then it may be involved in the pro-inflammatory effects of H_2S during sepsis. Moreover, we have shown that as O_2 levels fall, H_2S oxidation is reduced. The development of tissue hypoxia during sepsis would lead to decreased H_2S oxidation which may allow H_2S to accumulate to toxic levels and, possibly, enter the systemic circulation and contribute to systemic cardiovascular dysfunction.

The contention that H_2S contributes to hepatic hypoxia is muddled because H_2S is a vasoactive molecule which may affect DO_2 [48]. The main vasoregulatory action of H_2S occurs via activation of K_{ATP} channels in vascular smooth muscle cells (VSMCs) which leads to relaxation of resistance vessels [148]. The vasodilatory effects of H_2S may increase hepatic tissue perfusion and O_2 delivery which could mitigate the effect of H_2S oxidation on hepatic O_2 levels during sepsis. A vasodilatory effect of H_2S on the hepatic vasculature was first demonstrated by Fiorucci et al. using livers isolated from normal and cirrhotic rats [33]. They found that H_2S attenuated the vasopressor effects of norepinephrine which was independent of NO. Portal hypertension resulting from an increase in intrahepatic resistance is the main complication during chronic conditions like fibrosis and cirrhosis [7]. The vasodilatory effect of H_2S on the hepatic vasculature would be beneficial by decreasing hepatic resistance and attenuating the development of portal hypertension. While similarities exist between vascular dysfunction in cirrhosis and sepsis, the effect of H_2S on hepatic resistance during sepsis has yet to be investigated.

Unlike the systemic circulation, where tissue perfusion is primarily regulated by VSMCs surrounding pre-capillary resistance vessels (arterioles), hepatic perfusion is regulated at pre-sinusoidal as well as sinusoidal sites [11]. The modulation of pre-sinusoidal regulatory sites is similar to other vascular beds. In these vessels, vascular dysfunction following inflammatory stress is characterized by hyporeactivity to catecholamines [97]. Ample evidence suggests that a second functionally important regulatory site of hepatic perfusion in the liver is at the level of the sinusoid, particularly following inflammatory stress [25, 63, 98, 112]. The primary modulator of sinusoidal resistance is the hepatic stellate cell (HSC) [62, 146]. These specialized pericytes contract in response to vasoactive molecules including endothelins, eicosanoids, and angiotensin II [9, 49, 60]. Inflammatory stress is associated with the activation of HSCs which leads to a myofibroblast-like phenotype and enhanced contraction in response to vasoconstrictors, particularly endothelin-1 (ET-1) [105, 107]. Moreover, inflammatory stress leads to an uncoupling of ET-1/ET_B binding and eNOS activation in SECs due, in part, to an increase in the inhibitory protein caveolin-1 [67, 81, 113]. The resulting imbalance in the antagonistic relationship between vasodilators and vasoconstrictors leads to sinusoidal hyperconstriction which is a main cause of increased intrahepatic resistance in cirrhosis and sepsis. Therefore, we investigated the effect of H_2S on intrahepatic vascular responses during infusion of the α_1 adrenergic agonist, phenylephrine (PE), and ET-1 during sepsis.

Infusion of PE or ET-1was associated with an increase in portal pressure in livers isolated from control rats, which is indicative of an increase in total intrahepatic resistance in isolated livers during constant flow perfusion. An increase in intrahepatic

resistance is the summation of changes in resistance at both pre-sinusoidal and sinusoidal regulatory sites. Previous work from our lab demonstrated that PE and ET-1 exert their vascular effect at different sites [146]. Using *in vivo* microscopy, portal infusion of ET-1 resulted in an increase in portal pressure and sinusoidal constriction which colocalized with HSCs. While PE infusion increased portal pressure, no effect was observed in the sinusoids, suggesting that PE acts only at pre-sinusoidal regulatory sites, while ET-1 acts at sinusoidal and pre-sinusoidal sites [146].

Since microvascular dysfunction during sepsis occurs at both locations, it is important to consider the effects of H_2S in the presence of PE and ET-1. In agreement with previous studies from our lab, LPS treatment attenuated the vasoconstrictive effect of PE, whereas LPS potentiated the vasoconstrictive effect of ET-1 [94, 97]. During endotoxemia, VSMCs in portal venules become hyporesponsive to PE due to an increase in the production of vasodilatory molecules, such as NO [97]. In agreement with Fiorucci et al study, we showed that addition of the vasodilator H_2S attenuated the vasoconstrictive effect of PE in normal rats and nearly abolished the effect in LPS-treated rats. LPS treatment had the opposite effect on ET-1-induced vasoconstriction. The hepatic vasculature was hypersensitive to the vasoconstrictive effect of ET-1 during endotoxemia; however, we observed no effect of H_2S on ET-1-induced vasoconstriction in control or LPS-treated livers, suggesting that H_2S may not exert a vasoregulatory function in the sinusoids.

In vitro experiments often require large amounts of exogenous H_2S to demonstrate a biological effect which may not be reflective of *in vivo* levels [36, 138]. Therefore, we used the inhibitor of CSE, dl-propargylglycine (PAG) [126], to determine

the effect of endogenous H_2S during PE and ET-1 infusion. PAG treatment 30 minutes prior to liver isolation was associated with a significant increase in PE-induced increases in portal pressure. In septic and endotoxemic rats, arterial H_2S concentration is negatively correlated with blood pressure leading to the suggestion that H_2S contributes to the systemic hypotension [50]. Our findings tend to support this hypothesis and may indicate that the inhibition of endogenous H_2S has potential as an approach to treat diffuse hypotension during sepsis. However, a study by Collin et al showed that PAG treatment did not prevent circulatory failure in endotoxemic rats [26]. Interestingly, PAG treatment did prevent liver damage, suggesting that the deleterious effect of endogenous H_2S during sepsis may be liver specific.

The effect of PAG treatment on ET-1 infusion is more complex. PAG potentiated the effect of ET-1 infusion in control animals; however, PAG had no effect on the response to ET-1 in endotoxemic livers. Since ET-1 acts at both presinusoidal and sinusoidal sites, the increased sensitivity to ET-1 in control animals may occur at either or both sites. Therefore, it is possible that an increase in vascular resistance at pre-sinusoidal sites by PAG, similar to PE, could be counteracted by the vasodilatory effect of PAG in the hepatic sinusoid, resulting in no net change in total intrahepatic resistance.

In support of this scenario, we observed a significant improvement in O_2 consumption following PAG treatment in LPS-treated rats. ET-1 treatment stimulates glycogenolysis in isolated hepatocytes which results in an increase in O_2 consumption [111]. In our study, infusion of low concentrations of ET-1 caused an increase in O_2 consumption in control livers. At high concentrations, ET-1-induced vasoconstriction significantly impairs O_2 delivery to hepatic tissue and decreases O_2 consumption. In LPS-

treated rats, infusion of low concentrations of ET-1 decreases O_2 consumption which is consistent with a hypersensitization of the hepatic sinusoid to the vasoconstrictive effects of ET-1. PAG treatment prevented the decrease in O_2 consumption during infusion of low concentrations of ET-1 during endotoxemia, suggesting improved sinusoidal perfusion. Therefore, we hypothesized that H_2S causes sinusoidal constriction and contributes to the sensitization of the hepatic sinusoid to the vasoconstrictive effect of ET-1 during endotoxemia.

To test this hypothesis, we used intravital microscopy which allows for the direct visualization of the hepatic sinusoids and assessment of sinusoidal perfusion *in vivo*. Portal infusion of H_2S was associated with a modest increase in portal pressure and net sinusoidal vasoconstriction. Sinusoidal constriction is associated with an increase in heterogeneity of sinusoidal perfusion [57]. The increase in sinusoidal heterogeneity is characterized by the presence of constricted sinusoids juxtaposed with dilated sinusoids producing an increase in the heterogeneity of sinusoidal diameters. Following infusion of H_2S, there was an increase in the heterogeneity of sinusoidal diameters which is consistent with heterogeneous sinusoidal perfusion. Because sinusoidal vasoconstriction is a major component of hepatic microcirculatory failure, we investigated whether endogenous H_2S contributes to the sensitization of the hepatic sinusoid to ET-1 during endotoxemia.

Infusion of ET-1 was associated with a modest increase in portal pressure in control animals. The increase in portal pressure was associated with a slight reduction in sinusoidal diameter. In LPS-treated rats, the effects of ET-1 were significantly different. LPS treatment lead to a greater increase in portal pressure and caused a significantly

greater reduction in sinusoidal diameter including several occluded sinusoids. Moreover, there was a significant increase in the heterogeneity of sinusoidal diameters. Computer automated analyses of RBC flow through the sinusoids confirmed that sinusoidal flow heterogeneity was greater in endotoxemic animals compared to controls. PAG treatment alone was similar to controls in all aspects except for a greater number of non-perfused sinusoids; however, the reasons for this are unclear. PAG treatment 30 minutes immediately before microscopy led to a significant improvement in sinusoidal flow evidenced by less sinusoidal hyperconstriction, fewer non-perfused sinusoids, and less sinusoidal flow heterogeneity following ET-1 infusion. Accordingly, these changes in sinusoidal parameters were functionally important. Using NADH autofluorescence to assess hepatic O_2 availability, ET-1 infusion in LPS-treated animals significantly increase NADH fluorescence compared to controls. While PAG treatment had no effect on these parameters in control animals, it significantly improved all parameters following ET-1 infusion during endotoxemia.

For the first time, we show that H_2S exerts a vasoconstrictive effect on the hepatic sinusoid. Currently, the mechanism of H_2S-induced vasoconstriction remains unclear, but is likely independent of K_{ATP} channel activation [74]. In the sinusoid, the vasoconstrictive effect of H_2S is the result of a shift in the balance between dilators and constrictors which regulate HSC contractility. The rapid, transient action of H_2S in the sinusoid suggests an acute effect. In HSCs, Ca^{2+} dependent and independent pathways lead to contraction [69, 101]. The activation of K_{ATP} channels by H_2S is well documented; however, H_2S also interacts with calcium channels [85]. Additionally, H_2S may modulate the activity ion channels via sulfhydration of cysteine residues [86]. H_2S has been shown to increase

cytosolic [Ca^{2+}] in several cell types, including HUVECs and microglial cells [70, 82]. Therefore, it is possible that H_2S may directly cause HSC contraction via activation of Ca^{2+} channels and increased cytosolic [Ca^{2+}].

H_2S causes vasoconstriction in isolated aortic rings by inhibiting the accumulation of cAMP. In HSCs, the Ca^{2+} independent pathway, in particular the Rho/ROCK pathway, has greater significance on contractility than in smooth muscle cells [61, 120]. Accumulation of cAMP inhibits the Rho/ROCK pathway in HSCs and decreases their contractility in response to ET-1 [118]. Moreover, the sensitivity of ET_A receptors to ET-1 is decreased after cAMP accumulation [104]. Therefore, H_2S may produce vasoconstriction in the sinusoid by inhibiting cAMP accumulation in HSCs.

Our lab has previously focused on the importance of NO in hepatic microvascular dysfunction during sepsis. Several studies have demonstrated a complex relationship between hydrogen sulfide and NO. *In vitro* studies show an interaction between H_2S and NO to form a vasoinactive nitrosothiol. Thus, H_2S may cause vasoconstriction via reducing the bioavailability of NO. This is an intriguing hypothesis in the septic liver where H_2S synthesis is increased and impaired NO production contributes to vascular dysfunction. Several studies support the involvement of NO in H_2S-induced vasoconstriction. The relaxation of isolated aortic rings in response to acetylcholine is dependent on NO release by an intact endothelium [40]. Treatment with NaHS, an H_2S donor, attenuates the vasorelaxant effect of acetylcholine. The constrictor effect of H_2S is lost following removal of the endothelium which suggests an interaction with endothelial-derived NO [74]. A similar effect is observed using the H_2S precursor, L-cysteine which attenuates vascular relaxation due to acetylcholine and the nitric oxide

donor, sodium nitroprusside (SNP) [31, 55]. *In vivo,* the vasopressor effect of H_2S suggests an increase in peripheral resistance. Moreover, the vasoconstrictive effect of H_2S appears to be concentration dependent. While high concentrations of H_2S cause systemic hypotension, intravenous injection of low concentrations of H_2S increases mean arterial pressure. The vasopressor effect was abrogated by treatment with an inhibitor of nitric oxide synthase (NOS) implicating the involvement of NO. A decrease in NO bioavailability via an interaction with H_2S is likely a mechanism of importance in the hepatic sinusoid, particularly during sepsis due to impaired NO production.

CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

In conclusion, the aim of this dissertation was to investigate the effect of H_2S in the liver during sepsis. In the first section, we show that the liver is a central regulator of H_2S levels in the body. Using an isolated, perfused liver, we demonstrated a high capacity to oxidize H_2S at the expense of cellular O_2. The ability to clear H_2S from the perfusate was dependent on O_2 availability and remained a priority during sepsis. Furthermore, elevated levels of H_2S in the portal circulation decrease hepatic O_2 availability *in vivo*. In the second section, we show that H_2S differentially modulates the hepatic response to PE and ET-1, suggesting that H_2S may have a differential effect at pre-sinusoidal and sinusoidal blood flow regulatory sites. Lastly, in the final section, we show for the first time that H_2S is a constrictor in the hepatic sinusoid. Moreover, endogenous H_2S is a major contributor to the hypersensitization of the hepatic sinusoid to the constrictor effect of ET-1 during endotoxemia which contributes to tissue hypoxia.

Taken together, these findings support a deleterious role of H_2S during sepsis. We propose that the presence of H_2S in the liver during sepsis contributes to tissue hypoxia and hepatic dysfunction (Figure 38). The elevated levels of H_2S would require increased sulfide oxidation to prevent toxic accumulation at the expense of hepatic O_2. At the same time, the vasoconstrictive effect of H_2S in the hepatic sinusoid contributes to heterogeneous perfusion which impairs O_2 and further diminishes hepatic O_2 content. The resulting tissue hypoxia leads to hepatic dysfunction and cell death. Our study provides

evidence of a potential protective mechanism observed following inhibition of endogenous H_2S in sepsis.

Despite the findings presented herein, there are still several important questions that remain unanswered. The inverse relationship between H_2S and O_2 suggests that H_2S may affect the expression of the oxygen sensitive protein, hypoxia inducible factor 1α (HIF-1α). The level of HIF-1α is inversely related to cellular O_2 tensions [133]. Moreover, endotoxin increases HIF-1α in an O_2 independent manner and is pro-inflammatory [100, 110]. Therefore, it is possible that the pro-inflammatory effect of H_2S during sepsis is due in part to increased expression of HIF-1α. In preliminary experiments in mice given a moderate dose of endotoxin (10 mg/kg ip, 6 hr), PAG treatment significantly attenuated hepatocellular injury ($P<0.01$, Figure 39). While not statistically significant, PAG treatment demonstrated a trend towards a decrease in HIF-1α levels during endotoxemia (Figure 40), but had no effect on iNOS or HO-1 in endotoxemic mice (data not shown).

The effect of H_2S during hypoxia is not solely detrimental. In isolated hepatocytes, we hypothesized that H_2S would sensitize increase hypoxic stress by decreasing cellular O_2; however, we show that the H_2S donor GYY 4137 attenuated cell death during hypoxia in part due to a significant reduction in mitochondrial derived reactive oxygen species (ROS) (Figure 41). Therefore, further research is needed to characterize the effect of H_2S in the liver during hypoxia.

The effect of H_2S on NOS is also the subject of debate. In an acellular environment, H_2S inhibits the activity of all three isoforms of recombinant NOS [65-66]. On the contrary, H_2S increases NO production from eNOS in bovine arterial endothelial

cells suggesting a more complex function in physiological conditions [102]. Preliminary experiments from our lab show that Na_2S increases eNOS activity and NO levels in SECs. However, these results may be unique to the experimental conditions because Na_2S rapidly releases H_2S which escapes to the atmosphere within minutes of incubation at 37° Celsius. Therefore, it is important to perform these studies using the slow releasing H_2S donor GYY 4137. It is possible that H_2S may increase eNOS activity, but inhibits the accumulation of NO via the creation of a nitrosothiol. The importance of investigating the effect of H_2S on eNOS activity is underscored by the contribution of impaired eNOS activity to hepatic dysfunction during sepsis.

H_2S has shown promise as a potential therapy in several disease states, including acetaminophen induced hepatotoxicity, burn injury and ischemia/reperfusion injury [127]. Despite these promising findings, evidence suggests that H_2S contributes to the progression of sepsis. The purpose of this study was to investigate the effect of H_2S in the liver and its contribution to hepatic tissue hypoxia and microcirculatory dysfunction. The importance of these findings is underscored by the fact that sepsis is a common complication in several of the diseases that H_2S therapy is being proposed. The development of potential therapeutics that administers H_2S may need to be monitored closely to ensure that they do not exacerbate the progression of sepsis in this vulnerable patient population.

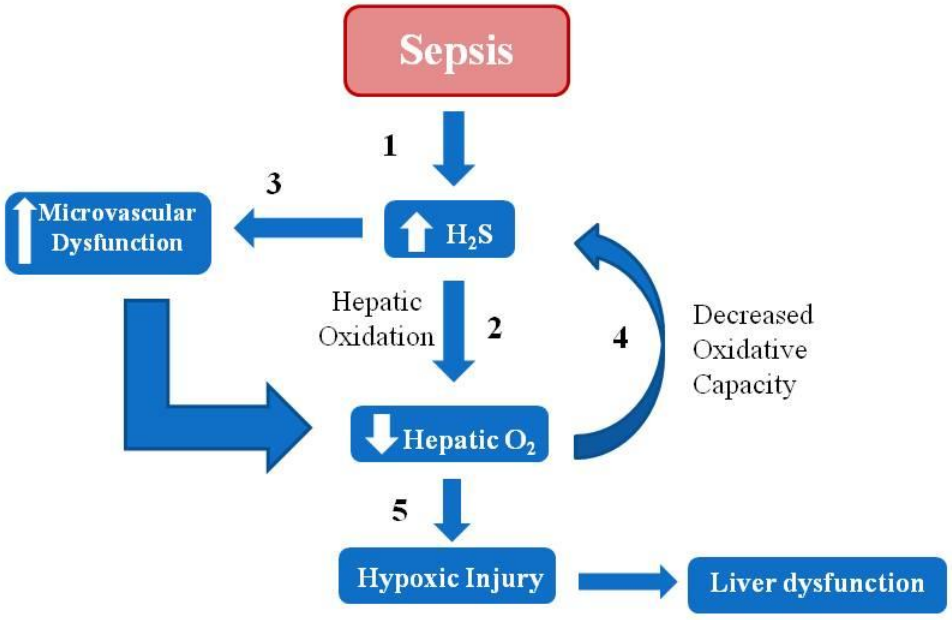

Figure 38: Proposed mechanism of the deleterious effect of H_2S during Sepsis. Sepsis increases hepatic exposure to hydrogen sulfide (1). The increased levels of H_2S require hepatic oxidation to prevent toxic accumulation. The oxidation of H_2S consumes molecular oxygen which reduces hepatic oxygen availability (2). Simultaneously, H_2S contributes to hepatic microvascular dysfunction which impairs hepatic O_2 delivery (3). The decrease in available oxygen reduces the oxidative capacity of the liver, producing a feed-back loop which further increases H_2S levels (4). The final result of this pathway is the potentiation of hypoxic stress which contributes to liver dysfunction during sepsis (5).

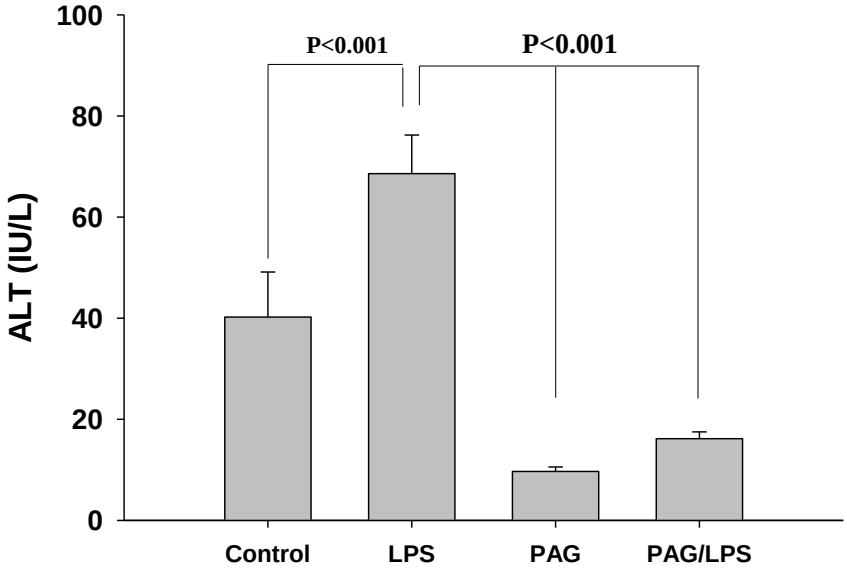

Figure 39: Effect of PAG treatment on serum alanine aminotransferase levels during endotoxemia**.** An intraperitoneal injection of PAG (50 mg/kg) or saline was given 30 minutes prior to LPS (10 mg/kg ip) or saline treatment. After six hours, blood was collected and analyzed for the ALT, a marker of hepatocellular injury. Data are presented as the mean ± SEM. Statistical analysis was performed using 2 way ANOVA with SNK *post hoc* test.

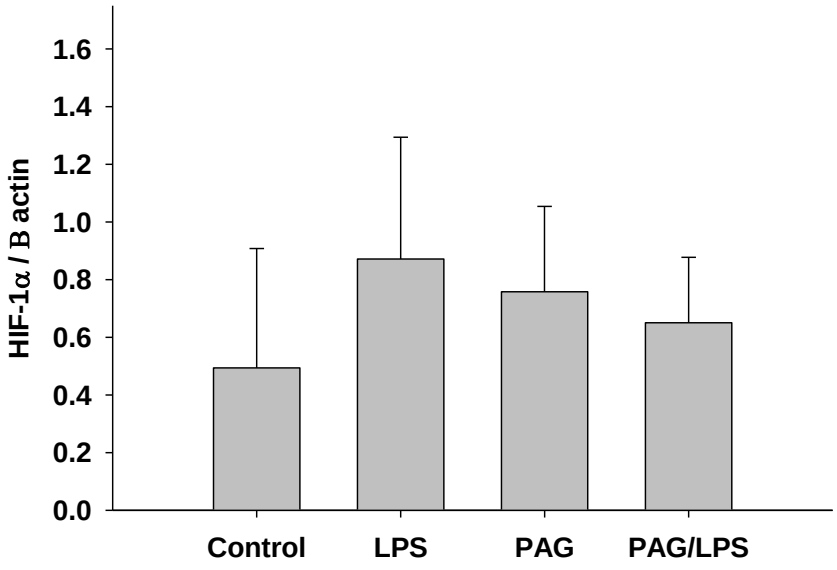

Figure 40: Effect of PAG treatment on hepatic HIF-1α levels during endotoxemia. HIF-1α levels were determined in liver homogenates from mice treated with LPS for 6 hours with or with PAG pretreatment. N=4.

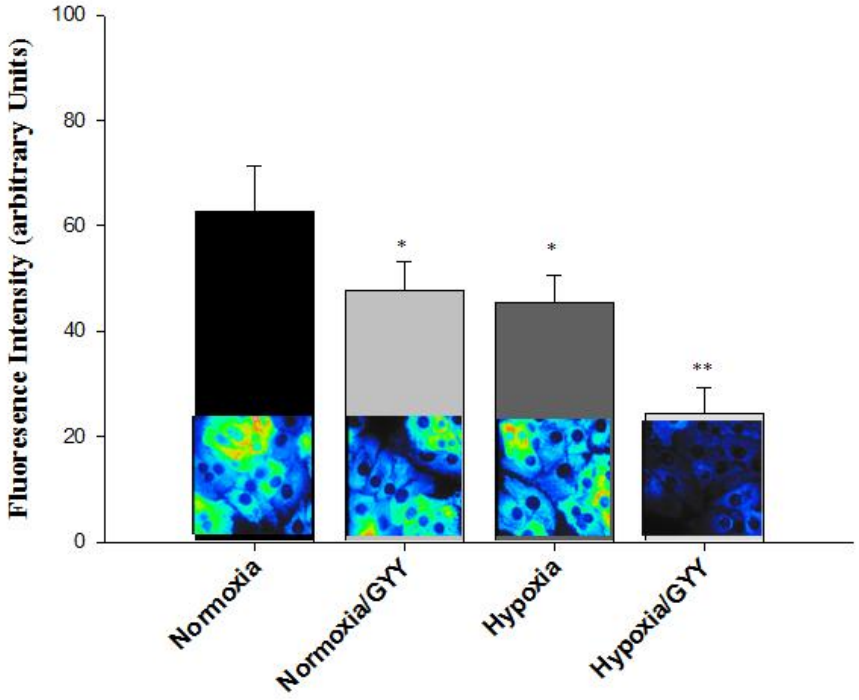

Figure 41: Effect of GYY 4137 on mitochondrial reactive oxygen species production in isolated rat hepatocytes during hypoxia. Hepatocytes were incubated under normoxic (20 % O_2) or hypoxic (< 3 % O_2) for one hour with or without the H_2S donor, GYY4137. Mitochondrial reactive oxygen species production was determined using the fluorescent dye Mitosox^{TM} (Molecular Probes, Grand Island, NY). Data are presented as means ± SEM of 5 separate experiments. Statistical analysis was performed using 2 way ANOVA with SNK *post hoc* test. *=P<0.05 vs. control, **=P<0.05 vs. Normoxia/GYY & Hypoxia .

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