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The liver as a central regulator of hydrogen sulfide

Eric J. Norris, Catherine R. Culberson, Sriram Narasimhan, and Mark G. Clemens

Department of Biology, University of North Carolina at Charlotte, Charlotte, NC

Abstract

The liver is likely exposed to high levels of H₂S from endogenous hepatic synthesis and exogenous sources from the gastrointestinal tract. Little is known about the consequence of H₂S exposure on the liver or hepatic regulation of H₂S levels. We hypothesized that the liver has a high capacity to metabolize H₂S and that H₂S oxidation is decreased during sepsis; a condition in which hepatic O₂ is limited and H₂S synthesis is increased. Using a non-recirculating isolated and perfused liver system, we demonstrated rapid hepatic H₂S metabolism up to an infusion concentration 200 μM H₂S. H₂S metabolism was associated with an increase in O₂ consumption from a baseline 96.7 ± 7.6 μmoles O₂/min/kg to 109 ± 7.4 μmoles O₂/min/kg at an infusion concentration of 150 μM H₂S (P<0.001). Removal of O₂ from the perfusate decreased H₂S 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 H₂S suggesting that H₂S oxidation remains a priority during sepsis. To test whether H₂S induces O₂ consumption *in vivo*, intravital microscopy was utilized to monitor the oxygen content in the hepatic microenvironment. Infusion of H₂S increased the NADH/NAD⁺ ratio (645 grey scale unit increase, P=0.035) and decreased hepatic O₂ availability visualized with Ru(Phen)₃²⁺ (439 grey scale unit increase, P=0.040). We conclude that the liver has a high hepatic capacity for H₂S metabolism. Moreover, H₂S oxidation consumes available oxygen and may exacerbate the tissue hypoxia associated with sepsis.

Keywords

H₂S; Hydrogen Sulfide; Hypoxia; Sepsis; liver

Introduction

For centuries, hydrogen sulfide (H₂S) has been known almost exclusively as a toxic gas associated with the characteristic smell of rotten eggs. However, recent evidence now demonstrates that H₂S is an endogenously produced biologically active gaseous molecule [1]. Numerous studies have identified H₂S as an important mediator in several biological systems including neurological, cardiovascular, and gastrointestinal systems [2-5]. The role of H₂S in inflammation remains poorly defined [6-11]. H₂S 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 [12-13]. Additionally, the synthesis of H₂S by the microflora of the gastrointestinal

Correspondence: Mark Clemens, Department of Biology, University of North Carolina at Charlotte, 9201 University City Boulevard, Charlotte, NC, 28223, Tel: 704(687)-8675, Fax: 704(687)-3128, mgclemen@uncc.edu.

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system provides an exogenous source of H₂S that may enter the portal circulation [4]. The liver is uniquely positioned to be exposed to high levels of H₂S; 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 H₂S 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 H₂S [14]. Therefore, we propose that the liver, as a consequence of its location, is a key regulator of H₂S levels by maintaining a high capacity for H₂S clearance from the circulation

Despite extensive research, sepsis remains a significant clinical problem today accounting for 1.3 percent of all hospitalizations [15]. Sepsis is associated with an inflammatory cascade resulting in hepatic dysfunction [16]. Failure of the hepatic microcirculation results in inadequate perfusion resulting in heterogeneous oxygen distribution and tissue hypoxia [17-18]. Several studies have demonstrated cellular metabolism of H₂S via oxidation in the mitochondria [19-20]. The oxidation of H₂S 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, H₂S 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 H₂S on the hepatic microenvironment, particularly during pathological conditions, such as sepsis, where oxygen availability is limited and H₂S synthesis is increased [10]. The role of H₂S during sepsis is still unclear and a better understanding is vital for the potential development of targeted therapeutic intervention [21]. Therefore, the aim of the present study was to assess the capacity of the liver to metabolize H₂S with particular emphasis on the relationship between H₂S oxidation and hepatic oxygen availability. The study was also designed to investigate whether the capacity for H₂S 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 H₂S clearance and hepatic oxygen availability.

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 [22]. 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 Krebs-Henseleit buffer [NaCl 118.95mM, MgSO₄ 1.2mM, KH₂PO₄ 1.2 mM, KCl 4.65 mM, NaHCO₃ 25 mM, EDTA 0.1 mM, CaCl₂ 2.5mM, C₃H₆O₃ (lactic acid) 5mM, C₃H₃O₃Na (pyruvic acid) 1 mM, C₆H₁₂O₆ (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 Krebs'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 O₂ consumption during the stabilization period were considered satisfactory preparations and used in future experiments.

Na₂S preparation

The H₂S donor Na₂S (Sigma-Aldrich, St. Louis, MO) was used in all experiments. Na₂S completely dissociates in water to the weak conjugate base S²⁻ which combines with available H⁺ ions to establish an equilibrium between H₂S $\rightleftharpoons$ HS⁻ + H⁺ at physiological pH. It remains unclear which form of H₂S is biologically active. For clarity, the term H₂S will be used to represent all sulfide added to the system from Na₂S. Na₂S was dissolved in the appropriate buffer immediately before use as H₂S 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.

H₂S clearance

Following the stabilization period, H₂S was infused into the influent at a rate of 1% of the of total flow rate resulting in final H₂S concentrations between 0-500 μ M in 50 μ M increments. Preliminary experiments demonstrated a rapid equilibration H₂S concentration in the perfusate. Thus, infusion of H₂S 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 H₂S infusion began. Samples were immediately transferred into 1.5 ml eppendorf tubes containing 150 μ l of 1% zinc acetate to trap the H₂S in solution. All samples were frozen and stored at -80 Celsius overnight before the H₂S assay.

For experiments requiring different levels of buffer oxygenation, Krebs's buffer was bubbled with a combination of gases (N₂, O₂, CO₂) 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 H₂O 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 [23]. Briefly, the excised heart was quickly transferred to a petri dish containing ice cold Krebs's buffer. The aorta was quickly cannulated and secured into position with suture material. Warm, oxygenated Krebs'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 H₂S 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 Krebs'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 μ M H₂S. 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 μ M. This concentration was chosen for comparison of gluconeogenesis and vasoactivity between control and the experimental H₂S group.

Gluconeogenesis assessment

Glucose production from isolated perfused livers was measured using effluent samples collected and stored at -80°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°C and then read at 450nm on a Beckman Spectrophotometer. Glucose concentrations were calculated from a standard curve of known values.

H₂S assay

75 μ l samples were immediately placed in 150 μ l of 1% zinc acetate to trap H₂S in solution. To this solution, 133 μ l of 20 mM N,N- dimethyl-p-phenylenediamine sulfate (dissolved in 7.2 M HCl) and 133 μ l 30 mM FeCl₃ (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 μ l of 10% trichloroacetic acid was followed by centrifugation (10,000g, 5 min 4° Celsius) to precipitate proteins. Absorbance was measured at 670 nm. H₂S 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 H₂S clearance was assessed as previously described.

Intravital Microscopy

Fasted rats were anaesthetized using sodium pentobarbital (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 H₂S 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 pentobarbital 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 $\mu\text{l}/\text{minute}$ for 10 minutes to determine the effect of infusion on hepatic oxygen distribution. Short acting alpha adrenergic agonist phenylephrine (0.15 $\mu\text{moles}/\text{min}/\text{kg}$) 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 $\mu\text{moles}/\text{min}/\text{kg}$ for 10 minutes to achieve an estimated plasma concentration of 200 μM . A 10 minute recovery period with saline infusion immediately followed H_2S infusion.

Assessment of oxygen distribution *in vivo* was performed as previously described [24]. 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 $\pm$ 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 $P < 0.05$.

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 Krebs's buffer and subjected to increasing infusion concentrations of the H_2S donor compound Na_2S to achieve final perfusate H_2S concentrations of 50-500 μM . The inflow and outflow perfusates were collected and sampled for H_2S levels. The H_2S clearance ratio (outflow perfusate $[\text{H}_2\text{S}]/\text{inflow perfusate } [\text{H}_2\text{S}]$) was used as a measure of the hepatic ability to clear H_2S at each concentration. Perfusion with Krebs'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 1A). Fifty percent H_2S clearance was observed at an infusion concentration of $303 \pm 28 \mu\text{M}$. At H_2S perfusate concentration greater than 400 μM , an almost complete inhibition of H_2S clearance was observed (Figure 1B). 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 (0-200 μM). In isolated hearts, a linear correlation exists between the concentration of H_2S in the inflow perfusate and outflow perfusate (Figure 2,

$R^2=0.979$, $P<0.001$). 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 $\mu\text{moles O}_2$ / min / kg body weight when perfused with Krebs'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 ($P<0.001$) $\mu\text{moles O}_2$ / min / kg body weight respectively (Figure 3A). 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 3B). At pathological H_2S concentrations greater than 250 μM , an increasing inhibition of oxygen consumption was observed with O_2 consumption being 74% of maximal consumption at 300 μM and 46% of maximal consumption at 500 μM . The requirement of O_2 for H_2S metabolism was tested by altering the oxygen content of the perfusate. Krebs'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 4). 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 Krebs'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 (50 μM) 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 (5 μM) and gluconeogenic substrates lactate (5mM) and pyruvate (1mM). After a 20 minute stabilization period with incomplete Krebs's buffer, isolated livers were perfused with complete Krebs's (addition of lactate and pyruvate) buffer containing phenylephrine. Immediately before addition of complete Krebs's buffer, baseline levels for portal pressure (3.96 ± 0.19 mmHg), oxygen consumption (1.824 ± 0.165 $\mu\text{moles O}_2$ / min / g liver wet weight), and glucose production (0.254 ± 0.11 $\mu\text{moles glucose}$ / min / g liver wet weight) were recorded. Addition of complete Krebs'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 5A). 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 ($P<0.001$)

$\mu\text{moles} / \text{min} / \text{g}$ liver wet weight ($P < 0.001$) following stimulation with phenylephrine and gluconeogenic substrates (Figure 5B). 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 5C, $P < 0.05$). 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 $196 \pm 35 \mu\text{M}$ respectively (Figure 6A). Sham treated livers reached a maximal H_2S clearance rate of $17.2 \pm 1.6 \mu\text{moles} / \text{min} / \text{kg}$ body weight at an infusion concentration of 132 μM (Figure 6B). CLP treated livers had a maximal H_2S clearance rate of 16.9 ± 1.9 $17.2 \pm 1.6 \mu\text{moles} / \text{min} / \text{kg}$ 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 ($< 150 \mu\text{M}$) concentrations and a decrease in oxygen consumption at high H_2S levels ($> 200 \mu\text{M}$) (Figure 6C).

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 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 $\text{Ru}(\text{Phen})_3^{2+}$ (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 $\text{Ru}(\text{Phen})_3^{2+}$ 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 7A) and $\text{Ru}(\text{Phen})_3^{2+}$ (439 grey scale unites, $P = 0.040$, Figure 7B) following 10 minutes of H_2S infusion, indicating that H_2S oxidation reduces the PO_2 in the hepatic microenvironment.

Discussion

Originally known for its cytotoxicity and pungent odor, H₂S is now recognized, along with nitric oxide and carbon monoxide, as the third major gasotransmitter with important physiological roles in neurological [2], cardiovascular [3], and gastrointestinal function[4]. H₂S 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, [12-13] resulting in physiological levels of H₂S reported to be between < 1 and 300 μM [14]. Exogenous H₂S is synthesized during the normal cellular metabolism of microflora in the gastrointestinal system and may enter the portal circulation [4]. As the major recipient of gastrointestinal blood flow, the liver is uniquely positioned to be subject to high levels of H₂S from a combination of endogenous and exogenous sources. Furthermore, H₂S synthesis is increased during pathological conditions including sepsis [10]. 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 H₂S from the circulation in an oxygen dependent manner. Additionally, we investigated whether hydrogen sulfide metabolism is altered during polymicrobial sepsis; a pathology in which H₂S levels are increased and limited oxygen availability is a contributing factor[18].

The hypothesis that the liver is capable of clearing H₂S via oxidation from the circulation is not new [19]. 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 H₂S 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 Krebs' 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 H₂S from the perfusate for over an hour in preliminary experiments (data not shown); a finding in agreement with the original H₂S clearance study.

Controversy exists over the physiologically relevant concentrations of circulating H₂S in blood with values ranging two orders of magnitude [14, 25]. 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 H₂S 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 H₂S may bind to hemoglobin or be metabolized by red blood cells into a physiologically inert form [26]. Therefore, we investigated the effect of H₂S infusion *in vivo* using intravital microscopy. Infusion of H₂S into the portal circulation via the splenic vein resulted in a significant decrease in hepatic oxygen content suggesting H₂S 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 H₂S is oxidized by the sulfide quinone reductase enzyme [20]. The study also demonstrated the capacity for H₂S 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 H₂S oxidation, evidence that organ function and cellular environment contributes to the capacity for H₂S oxidation. Similar results were

demonstrated in isolated and perfused kidneys and lungs [19]. Our results combined with those of Lagoutte et al indicate that the liver and colonic epithelial cells, cell types exposed to high levels of H₂S, are specialized for effective clearance of H₂S.

Lagoutte et al. also demonstrated that H₂S metabolism is a priority in mammalian cell types. Furthermore, oxidation of H₂S requires a significant amount of O₂ in a dose dependent manner. This finding was confirmed in our study. Low concentrations of H₂S (<200μM) resulted in a significant increase in oxygen consumption. As H₂S 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 [27]. The initial increase in O₂ consumption at high doses of H₂S gradually decreased until an almost complete inhibition of O₂ consumption was observed at 500μM. It is possible that the increase in oxygen consumption observed during infusion of H₂S could be a result of the vasodilatory effect of H₂S. In order to assess the vasodilatory effect portal pressure was monitored throughout infusion of H₂S. Portal pressure remained stable throughout the entire experiment indicating that H₂S 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 O₂ distribution which can be exacerbated during pathophysiological states such as sepsis [28]. Therefore, we tested whether alterations in O₂ availability affect H₂S metabolism. O₂ availability proved to be a requirement for H₂S metabolism as decreasing oxygen tension in the buffer resulted in a decreased ability to clear H₂S. In an extremely low O₂ environment the liver was almost incapable of clearing H₂S. Our findings are in agreement with the proposal that H₂S is a putative oxygen sensor as the levels of H₂S and O₂ are inversely related [29]. Moreover, a 2008 study by Whitfield et al demonstrated an accumulation of H₂S during periods of hypoxia that was rapidly reversed in the presence of oxygen [26]. Whether this increase in H₂S 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 [30], an increase in H₂S 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 H₂S 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, H₂S infusion resulted in an attenuation of the phenylephrine constrictor response indicating that H₂S was still biologically active [31]. Combined treatment with phenylephrine and H₂S led to greater O₂ consumption than phenylephrine alone. This was shown to be independent of gluconeogenesis as H₂S did not significantly alter phenylephrine induced increases in gluconeogenesis, an oxygen dependent process [32]. Thus, the increase in oxygen consumption with co-treatment with phenylephrine and H₂S is most likely a combination of increased hepatic perfusion as well as H₂S oxidation.

Hepatocellular dysfunction is known to occur in sepsis [16]. Loss of hepatocellular function leads to disease progression, however not all functions are affected equally [33]. 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) [17]. The decreased hepatocellular function combined with inadequate oxygen supply led us to hypothesize that the ability to metabolize H₂S would be greatly diminished in livers isolated from septic rats. Interestingly, our study demonstrated that metabolism of H₂S 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 [34]. 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 H₂S during the early inflammatory phase of sepsis.

In summary, the present study demonstrates that the liver has an important role in the regulation of H₂S levels in the circulation. While the debate continues regarding physiologically relevant levels of H₂S, we provide evidence that the rapid oxidation of H₂S during a single passage through the liver maintains low systemic circulating levels of H₂S. H₂S more likely acts as an auto- and paracrine signaling molecule [14]. However, especially during septic peritonitis, production of H₂S by intestinal bacteria is likely to increase H₂S levels in the hepatic portal blood. Excess H₂S production is removed from the circulation by the liver making it a key regulatory site for H₂S levels. The oxidation of increased levels of H₂S predisposes the liver to periods of tissue hypoxia. As hepatic O₂ levels drop, the capacity of the liver to metabolize H₂S decreases forming a detrimental positive feedback loop as more H₂S enters via the circulation. We have demonstrated that H₂S oxidation remains a priority during sepsis. H₂S oxidation could be especially detrimental during sepsis where increased H₂S levels during sepsis could exacerbate the limited oxygen available [35]. Tissue hypoxia contributes to hepatic injury through increased generation of reactive oxygen species [36-37]. Additionally, HIF 1 alpha, an hypoxia-dependent signaling molecule, is pro-inflammatory and may contribute to hepatic injury [38-39]. Ultimately, toxic levels of H₂S 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 H₂S presumably through cytochrome c oxidase inhibition [40]. Administration of H₂S has proven to provide therapeutic value in rodent models of ischemia/reperfusion injury [41], acetaminophen induced hepatotoxicity [7], and sepsis [5, 10, 41]. The complexity of H₂S during sepsis cannot be overstated as inhibition of H₂S production has proved therapeutic as well [10]. These discrepancies may be due to experimental parameters or time and dose of administration. Nevertheless, it is evident that H₂S has a role in pathophysiological states. We propose that the liver is unique in the role H₂S plays in pathophysiological states due to the priority given to modulate hydrogen sulfide levels in the circulation at the expense of hepatic oxygen availability.

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Fig. 1. Removal of H₂S 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. H₂S donor Na₂S was infused into the inflow perfusate to achieve final concentrations of 50-500 μM. Inflow and outflow perfusates were assayed for H₂S concentrations. **A)** The percent of H₂S cleared from perfusate is plotted against the calculated [H₂S] inflow. Data are presented as means ± SE 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. **B)** The total amount of H₂S cleared was determined by calculating the amount of H₂S cleared from the perfusate after a single pass through an isolated liver. Data are presented as means ± SE of three separate experiments (N=3).

Fig. 2. Isolated heart shows very limited capacity for H₂S clearance

An isolated and perfused heart was used for comparison to the liver's ability to clear H₂S from perfusate. Both organs were perfused at a constant flow rate with oxygenated Krebs buffer. H₂S donor Na₂S 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 H₂S concentration. Data are plotted as actual [H₂S] (μM) inflow versus actual [H₂S] (μM) outflow. Linear regression analysis was performed on final perfusate concentrations considered physiologically relevant (0-200μM).

Fig. 3. Hydrogen Sulfide metabolism produces a biphasic oxygen consumption response
Inflow and outflow perfusate oxygen tension was monitored during infusion of increasing H₂S concentrations (0-500μM) using an oxygen sensitive electrode. **A)** The change in oxygen tension in the inflow versus outflow was calculated as the amount of μmoles of O₂

consumed per minute per kg of body weight. One way repeated measures ANOVA was used for statistical analysis. Analysis for physiological concentrations (0-200 μ M) and pathological concentrations (250-500 μ M) were performed independently due to the biphasic response. Data are represented as means $\pm$ SE of three separate experiments (N=3). * P<0.01 compared to 0 μ M infusion. **B**) Individual tracings of the 5 minute H₂S infusion/ recovery cycle observed at Na₂S perfusate concentrations of 150, 250, and 350 μ M. Na₂S 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 μ moles O₂ consumed / min / kg body weight).

Fig. 4. Oxygen availability is a requirement for H₂S metabolism at physiological concentrations

To produce differential oxygen availability in an isolated liver, Kreb's buffer was continuously bubbled with a mixture of O₂, N₂, and CO₂ to achieve approximate final inflow O₂ content of 600, 300, 150, and 10 mmHg. Inflow and outflow perfusate was collected and sampled for H₂S content. Results are plotted as % H₂S clearance versus calculated [H₂S] (μM) inflow. Data are presented as means ± SE for three separate experiments (four in 600mmHg group) (N=3).

Fig 5. Hydrogen sulfide attenuates phenylephrine induced increase in portal pressure but not PE induced increase in gluconeogenesis

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 μ M H₂S. Data are means $\pm$ SE of 4 PE experiments and 3 H₂S 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. **A)** Portal pressure was monitored by a pressure transducer connected to the inflow during perfusion. **B)** Outflow perfusate samples from isolated livers were collected for determination of glucose output. **C)** Oxygen levels in the outflow perfusate were monitored to determine the effect of H₂S and phenylephrine on oxygen consumption.

Fig. 6. Cecal ligation and puncture does not affect hepatic H₂S clearance ability or H₂S associated changes in O₂ consumption in isolated livers

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 H₂S (50-500μM). Inflow and outflow perfusate were collected and assayed for H₂S content. **A)** Actual [H₂S] in the inflow perfusate is plotted against actual [H₂S] in the outflow perfusate. Data are presented as the percentage of H₂S cleared in a single pass through the liver. Data are presented as means ± SE of three separate experiments (N=3). **B)** Total H₂S clearance was measured from livers isolated from CLP and sham animals following infusion of Na₂S resulting in H₂S perfusate concentrations between 50-500μM. Data are presented as means ± SE of three separate experiments (N=3). **C)** Outflow perfusate from isolated livers from Sham and CLP treated rats was continuously monitored with an oxygen sensitive probe to determine the effect of H₂S on O₂ consumption. Data are presented as means ± SE of three separate experiments (N=3).

Fig. 7. H₂S infusion is associated with a decrease in hepatic tissue oxygen

Intravital microscopy was used to assess oxygen distribution in the hepatic microenvironment. 10 min saline infusion demonstrated stable oxygen distribution before H₂S infusion. H₂S was infused into the portal circulation for 10 minutes followed by a 10 minute saline infusion recovery period. **A)** 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⁺). **B)** Ru(Phen)₃²⁺ was infused into the circulation to serve as a direct oxygen sensor using intravital microscopy. Ru(Phen)₃²⁺ fluorescence (excitation 480 nm, emission 625nm) increases in the absence of oxygen. Grayscale was converted to pseudocolor with blue representing black (high O₂) and red representing white (low O₂). Micrographs (4x objective) of one experiment are representative of three individual experiments (N=3).