THE ROLE OF CYSTATHIONINE γ-LYASE AND HYDROGEN SULFIDE IN GLUCOSE TRANSPORTER GLUT1 EXPRESSION IN MACROPHAGES

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Abstract

Hydrogen sulfide (H_2S) is an endogenous gasotransmitter that regulates immune function and energy metabolism in macrophages. While it is known to impair mitochondrial respiration at high levels, recent studies have shown that it promotes aerobic glycolysis, highlighting its potential role in regulating macrophage inflammatory response. The investigation of the link between H_2S and glucose metabolism in macrophages is of particular significance due to the role of glycolysis in driving macrophage innate immune functions. Inflammation stimulates macrophages to increase glycolysis by upregulating the expression of Glut1, the primary glucose transporter in these cells. Nuclear factor (NF)-κB is a transcription factor that elicits inflammation by regulating the expression of pro-inflammatory cytokines and chemokines in response to stimuli such as lipopolysaccharide (LPS), a bacterial toxin. However, NF-κB controls Glut1 expression in macrophages during immune responses. The phosphoinositide 3-kinase (PI3k)/ protein kinase B (Akt) signaling also plays a role in regulating NF-κB activity and glucose metabolism. The precise impact of H_2S on the PI3k/Akt and NF-κB signaling pathways, as well as Glut1 expression in macrophages, remains poorly understood. The goal of my thesis research is to test for a link between endogenously produced H_2S and Glut1 expression during the pro-inflammatory response in macrophages. Small interfering (si)RNAs were used to knockout cystathionine γ-lyase (CSE) gene expression and block endogenous H_2S production in LPS-stimulated macrophages and the effect on LPS-induced Glut1 protein and mRNA expression was measured. Silencing CSE in LPS-stimulated macrophages reduced Glut1 mRNA levels suggesting that H_2S regulates Glut1 levels in inflammatory macrophages. Pre-treatment of macrophages with drug inhibitors targeting NF-κB or PI3k/Akt prevented LPS-induced Glut1 expression, implicating the signaling activity of these proteins as regulators of inflammation-

induced Glut1 expression. Silencing CSE decreased NF-κB activation in LPS-stimulated macrophages, suggesting that endogenous H_2S acts via NF-κB to supports Glut1 expression. To determine whether H_2S supports the LPS/NF-κB/Glut1 response, we treated LPS-induced macrophages with GYY4137, a slow releasing H_2S-donor molecule. Low levels of exogenous H_2S did not change Glut1 expression in LPS treated cells. However, high levels of H_2S inhibited NF-κB activation and Glut1 expression and increased Akt activation, suggesting an anti-inflammatory role of elevated H_2S levels. The anti-inflammatory effect of elevated levels of H_2S on LPS-induced NF-κB activation and Glut1 expression shows a marked difference from the pro-inflammatory impact of enzymatically produced levels, emphasizing the importance of distinguishing between the source and concentration of H_2S. These findings suggest that H_2S plays a role in inflammatory Glut1 expression through modulating NF-κB and Akt activity in macrophages.

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List of Abbreviations

Akt	Protein kinase B
ASS1	argininosuccinate synthase 1
ATP	Adenosine triphosphate
CBS	Cystathionine β-synthase
CO	Carbon monoxide
CSE	Cystathionine γ-lyase
DAMP	Danger associated molecular pattern
DMEM	Dulbecco's modified eagle medium
ELISA	Enzyme-linked immunosorbent assay
EM/EX	Emission / Excitation
ER	Endoplasmic reticulum
ERK	Extracellular receptor kinase
ETC	Electron transport chain
FBS	Fetal bovine serum
FL	Fluorescence
GABA	γ-aminobutyric acid
Glut1	Glucose transporter isoform 1
GPx	Glutathione peroxidase
GSH	Glutathione
HIF-1α	Hypoxia-inducible factor 1 alpha
H_2S	Hydrogen sulfide
IFN	Interferon

Iκβ	kappa gene enhancer in B-cells inhibitor
IKK	Iκβ kinase
IL	Interleukin
iNOS	Inducible nitric oxide synthase
M1	Classically activated macrophage
M2	Alternatively activated macrophage
JNK	c-Jun N-terminal kinase
KPE	Potassium phosphate EDTA buffer
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCP1	Monocyte chemoattractant protein-1
MFI	Mean fluorescence intensity
3-MST	3 mercapto sulfurtransferase
MYD88	Myeloid differentiation primary response 88
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NF	Nuclear factor
NO	Nitric oxide
NOX	NADPH oxidase
OXPHOS	Oxidative phosphorylation
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PE	Phycoerythrin
PFA	Paraformaldehyde
PI3k	Phosphoinositide 3-kinase
PLP	Pyridoxal 5′-phosphate
PPP	Pentose phosphate pathway
RET	Reverse electron transport
ROS	Reactive oxygen species
RT-qPCR	Real-time quantitative PCR
SD	Standard deviation
SOD	Superoxide dismutase
SQR	Sulfide quinone oxidoreductase
TCA	Trichloroacetic acid cycle
TLR4	Toll-like receptor 4
TNFα	Tumor necrosis factor-alpha
TSP	Transsulfuration pathway

Chapter 1 General Introduction

1.1. The role of hydrogen sulfide in modulating macrophage activation and glucose metabolism

Macrophages are cells in the innate immune system that provide the first line of defense against invading pathogens (Davies et al., 2013). Macrophages are classified broadly within two types of polarization states: classically activated (M1) and alternatively activated (M2) (Martinez and Gordon, 2014). Overall, M1 macrophages are effector cells that are induced by interferon (IFN)γ and lipopolysaccharide (LPS), produce pro-inflammatory cytokines and chemokines, as well as reactive oxygen and nitrogen intermediates, and play a role in host defense against pathogens (Herbst et al., 2011; Piedrafita et al., 2001; Thomas et al., 1997). Conversely, M2 macrophages are induced by cytokines, such as interleukin (IL)-4 and IL-10, and play a role in tissue repair, immune regulation, and homeostasis (Lavin et al., 2015). Recent studies have revealed that hydrogen sulfide (H_2S) acts as a potent inflammatory mediator in macrophages, modulating various activities, including migration, phagocytosis, and cytokine production, suggesting it as a promising therapeutic target for macrophage-mediated inflammatory conditions (Sun et al., 2021; Wang et al., 2020).

H_2S, initially identified as an environmental toxin, is now recognized to be a gasotransmitter that acts as an autocrine signaling molecule (Paul and Snyder, 2018; Sun et al., 2021). H_2S is synthesized in mammals via de-sulphuration reactions catalyzed by the enzymes 3-mercaptosulfurtransferase (3-MST), cystathionine β-synthase (CBS), and cystathionine γ-lyase (CSE), and the latter two are enzymes within the transsulfuration pathway (TSP). In basal conditions, TSP shunts homocysteine away from the methionine pathway and towards the cysteine biosynthesis pathway, ultimately leading to the production of glutathione (GSH), an

important antioxidant molecule in cells (McBean et al., 2015; Paul et al., 2018; Sbodio et al., 2019).

H_2S plays a role in cellular energy metabolism through its involvement in mitochondrial respiration, the Krebs cycle, and glycolysis (Carballal et al., 2021; Libiad et al., 2019; Vitvitsky et al., 2021). H_2S is a respiratory toxin at high concentrations but enhances the activity of the mitochondrial electron transport chain (ETC) and glycolysis at low concentrations (Libiad et al., 2019; Vitvitsky et al., 2021). The exact relationship between H_2S and Glut1 expression is not yet fully understood, but evidence suggests that H_2S may play a role in regulating Glut1 expression in macrophages during inflammation. Increased H_2S production via CSE supports the pro-inflammatory response of macrophages that contributes to inflammatory diseases in *in vivo* models of sepsis (Badiei et al., 2016, 2015; Gaddam et al., 2016). However, recent studies utilizing H_2S donors in similar models of induced sepsis note an opposite and anti-inflammatory role of this molecule (Chen et al., 2021; J. Li et al., 2021; T. Zhou et al., 2022). These conflicting results underscore the importance of considering the concentration of H_2S and its source in determining its function. Understanding the role of H_2S in regulating macrophage immune function and glucose metabolism through Glut1 expression could provide insight into potential therapeutic targets for inflammatory diseases. The following sections in chapter 1 outline the role of H_2S in modulating macrophage activation and glucose metabolism and highlight the gap in the literature regarding its role as an inflammatory mediator and regulator of cellular metabolism.

1.2. The regulation of the transsulfuration pathway: protein expression, substrate availability, and posttranslational modifications

H_2S is synthesized in mammalian cells predominantly by the TSP enzymes CBS and CSE that link the essential amino acid methionine to the GSH biosynthesis pathway via the metabolic

intermediate, homocysteine (Li et al., 2009; Beatty and Reed, 1980). The initial step in the TSP involves the condensation of homocysteine with serine to form cystathionine. This reaction is catalyzed by CBS and requires the cofactor pyridoxal 5′-phosphate (PLP) via a β-replacement reaction of serine by homocysteine forming cystathionine and H_2O (Chen et al., 2004). CSE is primarily responsible for converting CBS-derived cystathionine into cysteine in the presence of PLP. However, CSE is also known to exhibit substrate promiscuity and may catalyze a reaction between two cysteine molecules, producing H_2S (Singh et al., 2009). The majority of H_2S is generated in the TSP by CSE (Filipovic et al., 2018).

The first enzyme in the TSP, CBS, is constitutively expressed in cells, and is reported to be expressed in human primary macrophages and mouse-derived cell-line, J774A.1 macrophages (Bronowicka-Adamska et al., 2019a; Garg et al., 2006). The CBS protein contains a heme domain with a reported binding site for several ligands that induce allosteric regulation of the catalytic activity of CBS (Filipovic et al., 2018; Taoka et al., 2002). The heme domain exhibits affinity for several small molecules, including S-adenosylmethionine (Adomet) (Ereño-Orbea et al., 2013; Scott et al., 2004), as well as the gaseous signaling molecules, nitric oxide (NO) and carbon monoxide (CO), each of which inhibits CBS enzymatic activity (Taoka and Banerjee, 2001). The co-binding of Adomet can enhance the inhibitory effects of NO and CO on CBS (Vicente et al., 2016). Apart from this posttranslational regulation, CBS protein levels are downregulated in differentiated M1 macrophages of both mouse and human origin following exposure to interferon (IFN)γ and LPS, indicating that inflammation induces changes in TSP activity (Bronowicka-Adamska et al., 2019b; Garg et al., 2006). The decrease in CBS protein expression or its inhibition by NO or CO is hypothesized to cause depletion of cystathionine (Filipovic et al., 2018), suggesting a mechanism by which the TSP is rewired to decrease

cystathionine levels during the inflammatory response in macrophages (Fig 1.1). Inhibition of CBS activity or a reduction in its protein levels disrupts the biosynthesis of cystathionine, the essential substrate CSE utilizes for cysteine production. Under these conditions, CSE only catalyzes reactions for synthesizing H_2S, utilizing cysteine (Kabil et al., 2016).

CSE synthesizes cysteine from the essential amino acid methionine via the metabolic intermediate homocysteine (Filipovic et al., 2018). Cysteine derived from TSP contributes to the production of approximately one-half of the intracellular GSH pool in human liver cells (Mosharov et al., 2000). The transcription factor specificity protein (SP)1 maintains physiologic levels of gene transcript *cth*, the gene encoding CSE (Yang et al., 2011; Zhang et al., 2011). During inflammatory responses in macrophages, nuclear factor (NF)-κB induces the mRNA expression of CSE, leading to increased protein levels (Badiei et al., 2014) and an increase in H_2S synthesis (Zheng et al., 2013). In summary, the regulation of the TSP is a complex process that involves regulation of protein expression, substrate availability, and posttranslational modification of enzyme activities, all of which play a role in determining the pathway's response to cellular demands for cysteine versus H_2S synthesis.

1.3. The interplay between metabolism and the immune response in macrophages

NF-κB is a central regulator of inflammation, and its activation in macrophages can be triggered by a wide variety of stimuli, including microbial products, pro-inflammatory mediators such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and reactive oxygen species (ROS) generated during the immune response (Mulero et al., 2019). LPS is a PAMP ligand for the Toll-like receptor (TLR)4 binding domain on the cell membrane, which increases activity of NF-κB. Upon LPS binding, TLR4 dimerizes, and the molecular adapter myeloid differentiation factor 88 (MyD88) is recruited, leading to the activation of the Iκβ kinase (IKK) complex. Activated IKK

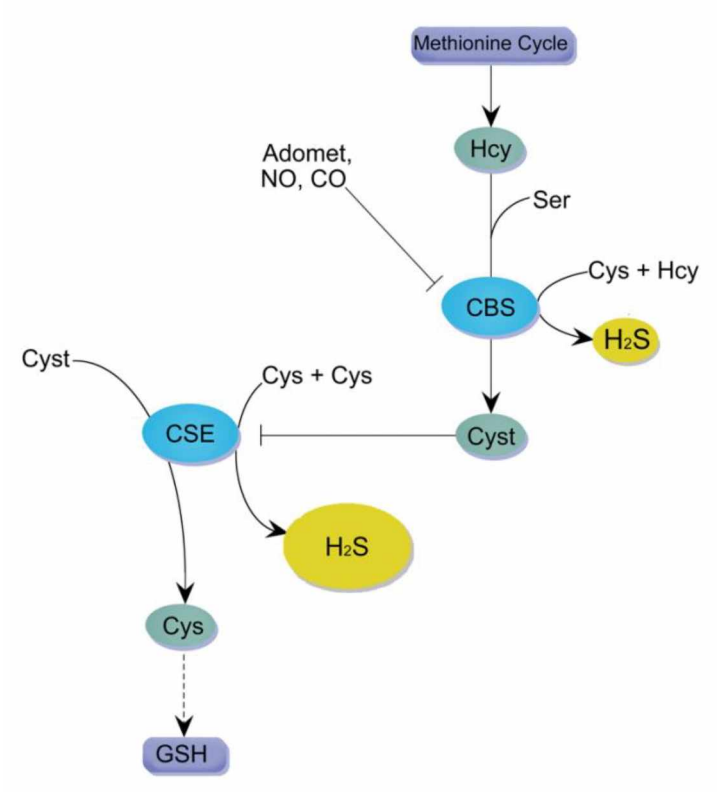

Figure 1.1: Proposed role of the transsulfuration pathway in producing H_2S

Homocysteine (Hcy) is utilized with serine (Ser) by CBS to produce cystathionine (Cyst). The heme domain within CBS is sensitive to allosteric regulation by ligands (NO and CO) whose concentrations change transiently in response to stimuli. Under basal conditions, Cyst outcompetes cysteine (Cys) utilization by CSE to generate Cys, which contributes to glutathione (GSH) synthesis. Heme-regulated switching of CBS activity or changes in CBS protein levels regulate CSE-derived H_2S versus cysteine synthesis. Homocysteine (Hcy), serine (Ser), cystathionine B-synthase (CBS), cystathionine (Cyst), cysteine (Cys), cystathionine y-lyase (CSE), hydrogen sulfide (H_2S), glutathione (GSH).

phosphorylates IκB (inhibitor of NF-κB), leading to its degradation, and subsequent dissociation from NF-κB. This then frees up the p65 subunit of NF-κB to translocate into the nucleus, where it binds to specific DNA sequences and activates the transcription of several target genes involved in immune and inflammatory responses, including tumor necrosis factor alpha (TNFα), IL-6, inducible nitric oxide synthase (iNOS), and IL-1β (Lu et al., 2008; Mulero et al., 2019).

Mechanisms are in place to quickly trigger metabolic reprogramming in macrophages upon immune activation. The increased expression of iNOS leads to the inhibition of mitochondrial respiration through the direct competition of NO with oxygen to inhibit cytochrome c oxidase, the terminal enzyme of the electron transport chain (ETC) (Everts et al., 2012). M1 macrophages use arginine to generate NO and citrulline via iNOS, while M2 cells, which do not express iNOS or produce NO, convert arginine to ornithine and urea via type 1 arginase (Shearer et al., 1997). During M1 macrophage immune activation argininosuccinate synthase (ASS1) is upregulated, which is an enzyme that increases arginine production to sustain NO levels. A study by Jha et al. (2015) revealed that in M1 macrophages, ASS1-derived NO disrupts mitochondrial oxidative phosphorylation (OXPHOS), requiring macrophages to enhance glycolysis to survive (Jha et al., 2015). Glycolysis quickly produces ATP; however, it generates less ATP per glucose molecule than OXPHOS. Nevertheless, glycolysis remains crucial in situations requiring rapid ATP production, such as inflammation.

In response to pro-inflammatory signals, macrophages undergo metabolic reprogramming, enhancing glycolysis to meet energy demands and support activating the immune response (Kelly and O'Neill, 2015). This metabolic reprogramming includes upregulation of the tricarboxylic acid (TCA) cycle and synthesis of arginine and succinate that support immune responses (Tannahill et al., 2013). M1 macrophages increase succinate levels, primarily sourced from glutamine-dependent anaplerosis and the GABA (γ-aminobutyric acid) shunt pathway (Tannahill et al., 2013). During inflammation, the accumulation of succinate in the cytosol, due to its transport from the mitochondria, can lead to the inhibition of prolyl hydroxylase activity and the stabilization of HIF-1α (Selak et al., 2005; Tannahill et al., 2013). Subsequently, HIF-1α upregulates IL-1β gene transcription (Tannahill et al., 2013). HIF-1α and

NF-κB collaborate in macrophages to promote glycolysis and the immune response. NF-κB can lead to the accumulation of HIF-1α by directly binding to its promoter and increasing its transcription (Cramer et al., 2003a; Rius et al., 2008). In addition, activation of HIF-1α in *ex vivo* macrophages infected with bacteria is dependent on IKKβ-responsive NF-κB (Rius et al., 2008). HIF-1α and NF-κB regulate gene transcription of *slc2a1*, which encodes the primary glucose transporter, Glut1, in macrophages (Cramer et al., 2003b; Freemerman et al., 2014; Obaid et al., 2021). Glut1 protein expression is induced to support M1 polarization following LPS stimulation (Freemerman et al., 2014; Obaid et al., 2021). Upon activation, M1 macrophages increase glucose uptake and utilization driving increased ROS by NADPH oxidase activity to combat invading bacteria (Sheppard et al., 2005). To maintain redox balance during M1 polarization, the glycolysis-PPP (pentose-phosphate pathway) axis supports the enhanced production of NADPH used by both NADPH oxidase (NOX) for ROS production, and glutathione reductase that maintains reduced glutathione (GSH) pools to counteract excessive superoxide production (Sheppard et al., 2005). Upregulation of Glut1 is triggered following LPS stimulation and leads to an increase in the production of NOX-derived ROS, driving inflammation (Freemerman et al., 2014). Even in the absence of LPS, transfection to induce the overexpression of Glut1 in RAW264.7 mouse macrophages increased PPP activation and ROS and pro-inflammatory cytokine levels, demonstrating the regulatory role of Glut1 in macrophage immune activation (Freemerman et al., 2014). Overall, the regulation of cellular metabolism is crucial to control macrophage activity, making it a promising target for developing therapies to treat inflammatory diseases.

1.4. Phosphoinositide 3-kinase / protein kinase B signaling pathway regulates cellular metabolism in macrophages

PI3k (phosphoinositide 3-kinase) is an enzyme that phosphorylates phosphatidylinositol lipids in the cell membrane, generating second messenger molecules that activate downstream signaling pathways. Akt (protein kinase B) is a serine/threonine kinase and a key downstream effector of PI3K signaling. Akt is activated by phosphorylation by PI3k and functions to promote cell survival, growth, and metabolism by phosphorylating and regulating a variety of downstream targets (Kane et al., 1999; Vergadi et al., 2017). Macrophages stimulated by LPS trigger the activation of PI3k signaling (Luyendyk et al., 2008), and PI3k activities are suggested to negatively regulate TLR signaling and prevent overactive immune stimulation of macrophages (Fukao and Koyasu, 2003). Such regulatory activities of PI3K/Akt signaling are important as they impact the crucial balance between pro-inflammatory and anti-inflammatory responses. The PI3K/Akt signaling pathway also regulates the expression and trafficking of Glut1, which is vital for the metabolic function of macrophages (Cheng et al., 2014; Covarrubias et al., 2015). Akt, a downstream effector of PI3k, can activate HIF-1α, which induces the expression of Glut1 and enhances glucose metabolism (Cramer et al., 2003; Joshi et al., 2014; Li et al., 2018). The trafficking of Glut1 is an important regulated aspect of glucose uptake that is also stimulated by growth factors, apart from inflammatory signals. Overall, PI3K/Akt signaling regulates macrophage metabolism and function, and dysregulation of these processes can lead to immune dysfunction and contribute to the pathogenesis of various inflammatory diseases.

1.5. Mitigating cellular damage from oxidative stress: protective measures and the role of glutathione and hydrogen sulfide in antioxidant defenses during immune response

Cellular components are sensitive to the damaging effects of ROS and require increased protective measures to mitigate ROS-triggered DNA damage, mitochondrial and endoplasmic reticulum (ER) stress, and uncontrolled immune response (Lee et al., 2014; Ribas et al., 2014; McBean et al., 2015; Paul et al., 2018). The effect of oxidative stress on the NF-κB pathway depends on the cell type and context. In response to oxidative stress, NF-κB can induce the expression of several antioxidants (Lingappan, 2018). The levels of superoxide dismutase (SOD) (Das et al., 1995; Rojo et al., 2004) and glutathione peroxidase (GPx) (Schreiber et al., 2006), among others, are increased via NF-κB to protect cellular components from self-inflicted damage and contribute to a regulated immune response.

As the primary mechanism of antioxidant defense against ROS and electrophiles, GSH serves a crucial role in maintaining cellular redox homeostasis (Ribas et al., 2014). GSH is synthesized *de novo* through two ATP-dependent reactions catalyzed by γ-glutamylcysteine synthase and glutathione synthetase. The redox-active thiol (-SH) of cysteine in GSH is oxidized to GSSG when it reduces target molecules, and requires NADPH to restore its reduced form (Pompella et al., 2003). The CSE enzyme plays a crucial role in maintaining the levels of GSH by producing cysteine, the limiting substrate for GSH synthesis (Lee et al., 2014). H_2S also increases GSH levels by enhancing cysteine transporter activity (Kimura, 2015) and enhances γ-glutamylcysteine synthase activity, which increases γ-glutamylcysteine levels (Kimura and Kimura, 2004). NF-κB significantly upregulates CSE during inflammatory response in macrophages (Badiei et al., 2014), and CSE indirectly contributes to antioxidant systems by producing cysteine and H_2S (Sun et al., 2021).

1.6. The complex role of hydrogen sulfide in modulating cellular energy metabolism and inflammatory responses in macrophages

H_2S plays a key role in regulating cellular energy metabolism and redox homeostasis, which is important for regulating inflammation in macrophages, but its precise role on immune cell inflammatory response is not yet fully understood. Due to the susceptibility of respiration to H_2S toxicity at high levels, the di-flavin enzyme, sulfide quinone oxidoreductase (SQR), which is localized on the inner mitochondrial membrane, acts as a respiratory shield. H_2S attacks the disulfide bond in SQR, generating a persulfide charge transfer complex. This outer sulfane sulfur is moved to a thiophilic acceptor, with GSH as the primary sulfur acceptor, safeguarding ETC function from H_2S (Kumar et al., 2021; Landry et al., 2021). H_2S orchestrates regulatory pathways in cellular bioenergetics, modulating mitochondrial energy production and promoting glycolysis. Previously, it was reported that treatment with the H_2S-donor molecule, GYY4137, induced the expression of Glut1 by stabilizing HIF-1α under normal oxygen conditions in THP-1 macrophages (Lohninger et al., 2015). This same study also reported that prolonged exposure (24h) of unstimulated macrophage to exogenous H_2S decreased NF-κB activity. The role of endogenous H_2S to regulate inflammatory Glut1 expression in macrophages is poorly understood. Previously, endogenous H_2S was found to support the activation of NF-κB, whereby siRNA targeting CSE inhibited the activation of NF-κB and the production of pro-inflammatory cytokines after LPS stimulation (Badiei et al., 2014; Sen et al., 2012). Resolving the paradoxical role of H_2S in macrophage metabolic function, which appears to both support and attenuate inflammation, is crucial for a better understanding of its potential as a therapeutic target for inflammatory diseases.

1.7. Summary and hypotheses

Glucose metabolism supports macrophage transition to a pro-inflammatory response. Previous studies have demonstrated that the endogenous CSE/H_2S system supports NF-κB activation under LPS-induced inflammation in macrophages, and exogenous H_2S donor molecules are reported to influence these same systems. In addition, the activation of PI3K/Akt pathway by H_2S and its subsequent regulation of macrophage metabolism implies a new possible mechanism underlying the impact of H_2S on glucose metabolism. The interplay between exogenous and endogenous H_2S and glucose metabolism is an intriguing area of investigation. H_2S appears to create a favorable redox milieu for glycolysis, suggesting potential crosstalk. Nevertheless, the precise effect of H_2S on glut1 regulation and its controllers remains elusive. Therefore, we propose H_2S may affect the inflammatory potential of macrophages by modulating Glut1 expression.

The following hypotheses were tested:

(1) I hypothesize that CSE knockdown and loss of endogenous H_2S production in LPS-stimulated macrophages reduces Glut1 mRNA and protein expression, and that this is caused by reduced NF-κB and PI3k/Akt activity (Fig 1.2). To test this hypothesis small interfering (si)RNAs were used to block CSE expression and endogenous H_2S production in LPS-stimulated macrophages. Subsequently, mRNA and protein were extracted and siRNA-mediated knockdown of the CSE gene was confirmed by Western blot (protein) and qPCR (mRNA). The gene and protein expression of Glut1 was also determined by Western blot and qPCR to elucidate whether the CSE/H_2S system impact LPS-induced Glut1 expression. To determine the role of NF-κB and PI3k/Akt, protein levels of

phosphorylated (activated) subunit p65 of NF-κB and subunit Ser473 of Akt protein levels were measured by ELISA. Pharmacological inhibitors of NF-κB and PI3k/Akt activation were applied to macrophages to confirm the role of these proteins in LPS-induced Glut1 mRNA expression. Specifically, Bay11-7042 (targeting NF-κB) or LY294002 (targeting PI3k/Akt) treatment followed by LPS confirmed the role of these protein to upregulate Glut1 mRNA expression following LPS stimulation.

(2) I hypothesize that treatment with H_2S-donor molecule, GYY4137, in LPS-stimulated macrophages increases Glut1 mRNA and protein expression and is mediated by increased NF-κB and PI3k/Akt activity (Fig 1.2). To determine the role of exogenous H_2S in macrophage LPS-stimulated Glut1 mRNA and protein expression, various concentrations of GYY4137 were added to macrophages along with LPS. Various concentrations were used to elucidate whether an observed effect is concentration dependent. Differences in Glut1 protein and mRNA were measured by Western blot and qPCR, respectively. In addition, the NF-κB and PI3k/Akt activated subunits were measured by ELISA.

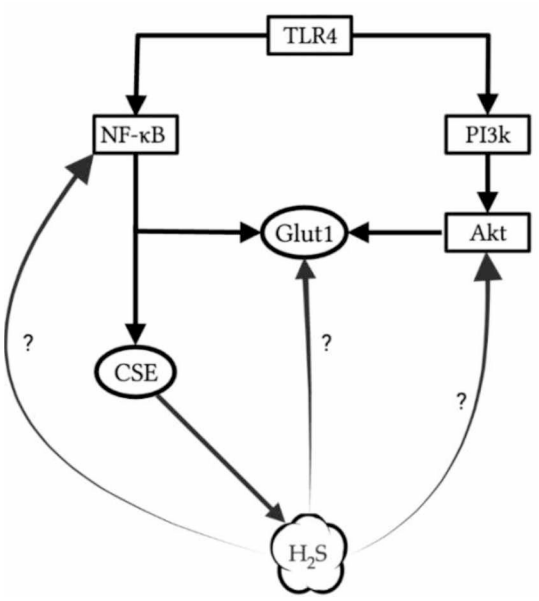

Figure 1.2: Proposed roles of H_2S-mediated Glut1 expression during immune stimulation

Toll-like receptor 4 (TLR4) signaling enhances glucose transporter Glut1 expression via phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) and nuclear factor (NF)-κB. The role of endogenously produced H_2S, by cystathionine γ-lyase (CSE) or exogenous H_2S by donor molecules are hypothesized to support Glut1 expression, and NF-κB and Akt activation may support this expression. The primary aim of this thesis proposes a link between H_2S and inflammation induced Glut1 expression.

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Chapter 2 Cystathionine γ-lyase and hydrogen sulfide modulates glucose transporter Glut1 expression via NF-κB and PI3k/Akt in macrophages during inflammation [1]

Macrophages are key regulators of the innate immune system, performing a crucial role in initiating and modulating inflammatory responses. Metabolic function powered by glucose transporter isoform 1 (Glut1) is necessary for macrophage activity during inflammation; however, the role of the novel gasotransmitter hydrogen sulfide (H_2S) in glucose metabolism remains unclear. The present study investigates the roles of cystathionine-γ-lyase (CSE) and its product, H_2S, in macrophage glucose metabolism to explore how H_2S acts as an inflammatory regulator in lipopolysaccharide- (LPS) induced macrophages. Our results demonstrate that LPS-treated macrophages increased Glut1 expression. This LPS-induced Glut1 expression is shown to be regulated via nuclear factor (NF)-κB activation and is associated with phosphatidylinositol-3-kinase PI3k/ Akt activation. Small interfering (si) RNA-mediated silencing of CSE decreased the LPS-induced activation of NF-κB and Glut1 expression, suggesting the CSE/H_2S system's important role in regulated metabolic reprogramming in macrophages. To test H_2S as an effector, we treated LPS-induced macrophages with GYY4137, an H_2S-donor molecule. Low levels of H_2S had a minimal impact; however, elevated levels showed a marked inhibitory effect upon LPS-induced NF-κB activation and Glut1 expression. The anti-inflammatory effect of elevated levels of H_2S on LPS-induced NF-κB activation and Glut1 expression shows a marked difference from the minimal anti-inflammatory impact of low levels, emphasizing the importance of distinguishing between endogenous production and pharmacologic administration. Overall, our study provides evidence that the CSE/H_2S system plays key roles in LPS-induced Glut1 expression and glucose metabolism by modulating NF-κB activation and suggests that this system regulates metabolic programming to alter the immune response.

[1] Extended version of: Cornwell, A., Fedotova, S., Cowan, S., Badiei, A., 2022. Cystathionine γ-lyase and hydrogen sulfide modulates glucose transporter Glut1 expression via NF-κB and PI3k/Akt in macrophages during inflammation. PLoS One, 17(12), e0278910. (See Appendix)

2.1. Introduction

Hydrogen sulfide (H_2S) is an endogenously produced inflammatory mediator increasingly recognized for its protective role in various inflammatory diseases (Deng et al., 2023; Jiang and Chen, 2022; Kaziród et al., 2022; Liu et al., 2022; Tian et al., 2022; J. Zhou et al., 2022). H_2S functions as a signaling molecule with diverse roles, including scavenging oxidants, modifying protein cysteine residues via persulfidation, and regulating energy production by redox interactions with mitochondrial and cytosolic metabolism and proteins (Castelblanco et al., 2020; Kimura, 2020; Li et al., 2005; Zhu et al., 2010). H_2S is produced in mammalian cells from L-cysteine predominately by cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS), and 3-mercaptopyruvate sulfotransferase (3-MST) (Li et al., 2009). Since its acceptance as a novel gaseous signaling molecule along with carbon monoxide (CO) and nitric oxide (NO), significant steps have been made to elucidate the physiological effects and role of H_2S in immune systems (Bhatia and Gaddam, 2021; Wallace et al., 2012).

A growing number of publications report on the cytoprotective effects of H_2S under various pathophysiologic conditions (Wallace et al., 2012; Wang et al., 2020). H_2S affects antioxidant systems and promotes anti-inflammatory functions (Sun et al., 2021). H_2S has been shown to attenuate lipopolysaccharide (LPS) induced formation of inflammatory mediators in murine RAW264.7 macrophages (Whiteman et al., 2010). H_2S is primarily generated in the transsulfuration pathway by CSE-catalyzed de-sulphuration of cysteine (Wallace et al., 2012; Zheng et al., 2013); however, CSE acts primarily on CBS-derived cystathionine to generate cysteine (Singh et al., 2009; Filipovic et al., 2018). Cysteine is the limiting substrate for glutathione (GSH), and its status affects antioxidant defense systems (Lee et al., 2014). Due to these activities, it is hypothesized that H_2S is primarily an anti-inflammatory molecule (Sun et

al., 2021). Despite this, the role of H_2S as a pro-inflammatory mediator that supports the immune systems has been implicated in contributing to macrophage activation and inflammatory responses (Wang et al., 2020).

Studies conducted *in vitro* indicate that H_2S contributes to macrophage inflammation by activating the ERK-NF-κB pathway (Badiei et al., 2014). However, in *in vivo* mouse model of sepsis, prolonged exposure and significantly increased levels of H_2S are reported to contribute to various acute (Badiei et al., 2016; Gaddam et al., 2016) and chronic (Muniraj et al., 2017) inflammatory diseases. Treatment with small interference RNA (siRNA) targeting the CSE gene reduced inflammation of macrophages in the liver and lungs of mice following cecal ligation puncture (CLP)-induced sepsis, indicating that CSE gene silencing with siRNA may be a promising therapeutic approach for sepsis (Badiei et al., 2016).

Macrophages require a distinct metabolic profile that depends upon and regulates their functional state. Pro-inflammatory activated macrophages stimulated by LPS reprogram cellular metabolism to support increased glycolysis and restrict mitochondrial oxidative phosphorylation (OXPHOS). Glut1 is the primary rate-limiting glucose transporter in macrophages and a critical regulator of macrophage inflammatory response (Freemerman et al., 2014; Obaid et al., 2021). The rapid increase in Glut1 during inflammation in macrophages drives increased glucose uptake and utilization to induce a reactive oxygen species (ROS) driven pro-inflammatory response (Freemerman et al., 2019). The PI3K/Akt signaling pathway plays a role in regulating Glut1 expression in macrophages. Akt, a downstream effector of PI3K, can activate the transcription factor HIF-1α, which induces the expression of Glut1 (Bai et al., 2009; Ozes et al., 1999; Vergadi et al., 2017). NF-κB and HIF-1α cooperatively promote glycolysis and immune response in macrophages, with NF-κB activating HIF-1α and regulating Glut1 expression, supporting M1

polarization after LPS stimulation (Freemerman et al., 2014; Obaid et al., 2021; Rius et al., 2008). Akt plays a crucial role in NF-κB activation by mediating NF-κB IKK phosphorylation, while HIF-1α transcriptional activation depends on IKK-responsive NF-κB (Bai et al., 2009; Rius et al., 2008; Vergadi et al., 2017).

In response to inflammation, proinflammatory macrophages undergo metabolic reprogramming, leading to an upregulation of Glut1 to meet the increased glucose demand. A previous study reported that GYY4137, an H_2S donor molecule, can increase Glut1 expression and HIF-1α stabilization in normoxic conditions, and exposure to 32 μmol/L H_2S for 24 hours was found to decrease NF-κB activation and TNFα levels. Previous studies have reported inconsistent measurements of endogenous concentrations of H_2S, with some suggesting microM concentrations, but recent studies using more sensitive techniques have found that the levels of endogenously produced H_2S are extremely low, measuring at approximately 10-70 nM in the murine liver, brain, heart, muscle, esophagus, and kidney (Levitt et al., 2011, Vitvitsky et al. 2012). Endogenous H_2S produced by CSE has been demonstrated to support NF-κB and proinflammatory responses in *in vitro* RAW264.7 macrophages and *ex vivo* mouse macrophages exposed to LPS (Badiei et al., 2015, 2014). The pro-inflammatory response in sepsis induced by cecal ligation puncture is mediated in part by H_2S synthesized by CSE in macrophages, and siRNA treatment targeting the CSE gene can effectively attenuate inflammation in the liver and lungs, as demonstrated by reduced myeloperoxidase activity and cytokine/chemokine levels (Badiei et al., 2016; Gaddam et al., 2016). The findings suggest that the effect of H_2S on macrophage function is complex and depends on the specific concentration and context in which it is present. Further research is needed to elucidate the molecular mechanisms underlying these

effects and to determine whether H_2S could be a potential therapeutic target for inflammation-related diseases.

In this study, we investigated the CSE/H_2S system's role in macrophage metabolic function during the immune response and examined the role of NF-κB and PI3K/Akt in this process. We hypothesized that CSE/H_2S system plays a critical role in inflammation-induced glucose metabolism due to previous evidence that crosstalk exists between H_2S and NF-κB and PI3K/Akt activities (Badiei et al., 2015, 2014; Gong et al., 2016; Zheng et al., 2013). Our study provides evidence that the endogenous CSE/H_2S system supports the pro-inflammatory expression of Glut1. In addition, our results support the conclusion that elevated levels of H_2S from H_2S-donor molecules attenuate LPS-induced Glut1. Our study implicates a role that H_2S levels may be modulated to regulate macrophage immune response.

2.2. Experimental methods

2.2.1 Cell culture

RAW264.7 (ATCC, Manassas, VA) murine macrophage cell line was cultured in DMEM (Gibco, Waltham, MA) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS; Cell Applications Inc., San Diego, CA), 100 units/ml penicillin, and 100 μg/ml streptomycin (Gibco) and maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were counted using a cell counter (Countess, ThermoFisher), seeded to 2.4×10^6 cells onto 6-well culture plates, and grown to confluence. After reaching 70% confluence, macrophages were ready for treatment.

2.2.2 Lipopolysaccharide treatment

Macrophages were stimulated with *E. coli*-derived LPS (100 ng/mL; Invitrogen, Waltham, MA) for 4 h. The concentration of LPS has been reported by other laboratories to

induce immune and pro-inflammatory responses in macrophages (Badiei et al., 2015, 2014; Whiteman et al., 2010; Zheng et al., 2013). After treatment, macrophages were harvested and used immediately to extract RNA or protein.

2.2.3 siRNA-mediated knockdown of CSE gene

Silencer Select pre-designed siRNAs (Ambion, Austin, TX) targeting the CSE mRNA and negative scramble control siRNA were used in the gene silencing experiments. CSE is the dominant H_2S-producing enzyme in several tissues, including macrophages (Kimura et al., 2012; Zhu et al., 2010); silencing CSE diminishes macrophage capacity to produce endogenous H_2S. Following manufacturer instructions for lipofectamine RNAiMAX (Invitrogen), cells were incubated with 5 pmol siRNA-lipofectamine complex for 24 h. After incubation, the medium was replaced, and cells were further treated.

2.2.4 H_2S-donor treatment

H_2S-donors were treated to cells simultaneously with LPS at different concentrations. The concentration of H_2S donor molecules tested (10, 100, 500 μM) was chosen based on previous work by Whiteman *et al.* (2010) to elucidate low and high pharmacologic concentration-dependent responses following LPS-induced stimulation of RAW264.7 cells. H_2S-donor treatment groups were treated with slow-releasing GYY4137 (GYY; 10 μM or 100 μM or 500 μM; Cayman Chem.) H_2S donors. GYY4137 rate of release is similar to the enzymatic production of H_2S (Li et al., 2008; Whiteman et al., 2010). Concentrations of 10, 100, or 500 μM GYY4137 achieve a steady state level of approximately 0.32, 3.2, and 16 μmol/L of H_2S for an over 24-hour period, respectively (Lohninger et al., 2015).

2.2.5 Protein extraction and Western blot

The treated macrophage cells were washed with ice-cold PBS and then lysed in RIPA cell lysis buffer, and 1% Halt protease inhibitor cocktail (Thermo Fisher). The resulting cell lysates were centrifuged for 20 min. at 20,000 g at 4 °C, and the protein concentrations in the supernatants were determined using a Pierce BCA protein assay kit (Thermo Fisher) (Smith et al., 1985). 20 μg proteins were loaded onto 10% SDS-PAGE gels, followed by electro-transfer onto nitrocellulose-membrane (Bio-Rad). The membranes were blocked in 1 × TBST (0.1% Tween-20, 20 mM Tris–Cl (pH 8.0), and 150 mM NaCl) containing 5% nonfat dry milk powder and then incubated with the primary CSE (1:1000 dilution, 12217, Proteintech), Glut1 (1:1000 dilution, 21829, Proteintech), and GAPDH (1:1000 dilution, 10494, Proteintech) antibodies overnight at 4°C. Membranes were washed 3 times (1 × TBST), then incubated with horseradish peroxidase-conjugated secondary goat anti-rabbit IgG H&L (1:10,000 dilution, 00001, Proteintech) antibodies for 1 h at room temperature, and then washed 3 times (1 × TBST). Lastly, immunoreactive proteins were detected using enhanced chemiluminescence detection kit (Bio-Rad). Band density was obtained using the Amersham imager 600 chemiluminescence auto exposure settings, the bands were quantified by Image J 1.8.0172 software (National Institutes of Health) and the protein levels in treated cells were normalized to untreated controls.

2.2.6 RNA extraction and RT-qPCR

Total RNA from cells was extracted using Trizol and chloroform reagents (Invitrogen, Waltham, Ma) following the manufacturer's instructions. RNA sample concentrations and purity were determined using Nanodrop One (ThermoFisher, Waltham, Ma). Primers were designed to span exon-intron junctions to ensure amplification of cDNA. First-strand cDNA synthesis was performed with 5 μg total RNA using Superscript III reverse transcriptase (Invitrogen, Waltham,

Ma) and random hexamers (IDT, Coralville, IA) and stored at −80 °C. qPCR was used to determine the relative expression of mRNAs on the QTower^3 system (Analytic-Jena, Thuringia, DE). PowerUp SYBR Green Mix (Applied Biosystems, Waltham, MA) was used with 250 nM forward and reverse primers, according to the manufacturer's instructions, in a 10 µL/384-well format following the standard SYBR green cycling mode. To compare the mRNA levels between different samples, the $2^{-\Delta Ct}$ (Schmittgen and Livak, 2008) method was used, and data were normalized to GAPDH. Experiments were run in triplicate; each sample represents three technical repeats. The sense and antisense primers were designed using the NCBI BLAST-Primer select tool (Table 2.1). Primers were designed to span exon/intron junctions to confirm mRNA specificity. To determine primer efficiency, serial dilutions of pooled sample cDNAs were diluted to produce an 8-point standard curve of averaged technical triplicates of Ct values vs. $\left(\frac{1}{dil.factor}\right)$. Next, the slope of the linear regression was used to determine the efficiency of the qPCR reaction, using the following equation: $E = \left(10^{\frac{-1}{Slope}} - 1\right) \times 100\%$. Primer efficiency between 90-110% were confirmed. Minus reverse transcription control (-RT control) was included in all RT-qPCR experiments to test for contaminating DNA (such as genomic DNA) from pooled RNA samples. Such a control contains all the reaction components except for the reverse transcriptase. A non-template control, lacking template DNA but including all other reaction components, was used to monitor for contamination or amplification from other sources were tested for each primer pair. Amplicon base-pair sizes were verified by agarose gel electrophoresis and compared with a standard DNA ladder.

Table 2.1: PCR primers

Gene	Forward (5′–3′)	Reverse (5′–3′)
Glut1	GATCTGAGCTACGGGGTCTT	TGTAGAACTCCTCAATAACCTTCTG
CSE	CAAAGCAACACCTCGCACTC	ATGCAAAGGCCAAACTGTGC
GAPDH	CGTCCCGTAGACAAAATGGT	GAGGTCAATGAAGGGGTC

2.2.7 Glutathione Level assay

GSH levels in macrophages were determined using a one-step fluorometric kit (Fluorometric-Green, ab138881, Abcam, Waltham, MA) according to the manufacturer's protocol. KPE (potassium phosphate–EDTA) buffer was prepared by adding 0.1 M phosphate buffer, mixture of 1.6 parts of solution A (99.96 mM KH_2PO_4 in dH_2O) to 8.4 parts of solution B (97.59 mM K_2HPO_4 in dH_2O) with pH adjusted to 7.5 and then EDTA sodium salt was added to a final concentration of 1.12 mM (Rahman et al., 2007). Following siRNA and LPS treatments, macrophages were removed from culture plates and counted to 10^6 cells per aliquot. Aliquoted cells were lysed in 0.5 mL KPE buffer with 0.1% Triton X-100 and 0.6% sulfosalicylic acid on ice; sulfosalicylic acid inhibits γ-glutamyltransferase activity and stabilizes GSH (Rahman et al., 2007). Cell lysates were then vortexed for 15 sec and centrifuged at 8,000 g for 10 min. The supernatants were transferred to pre-chilled microcentrifuge tubes. The concentration of total protein in each sample was determined by Pierce BCA protein assay kit. Samples were then mixed 1:1 with glutathione detection reagent to a final volume of 100 µL on 96-well plates and

incubated in the dark at room temperature for 30 min. Then, fluorescence intensity was measured at EX/EM of 490/520 nm, using the Biotek synergy H1 spectrophotometer. GSH level was calculated from the standard curve, normalized to the protein level, and expressed as μM GSH per mg protein.

2.2.8 Glut1 Surface level detection by flow cytometry

For immunofluorescence surface staining of macrophages, cells were suspended and fixed at a concentration of 10^6 cells per 100 μL in 4% paraformaldehyde for 15 min at room temperature. The cells were then washed with 1× PBS (3 times) and suspended (10^6 cells per 100 μL) in antibody dilution buffer (1X PBS containing 3% bovine serum albumin) with primary antibodies targeting Glut1 (1 μg/ml, 21829, Proteintech) for 1 h at 4 °C. Cells were then washed 3 times with 1× PBS followed by incubation with R-phycoerythrin R-PE, conjugated secondary antibody (1 μg/ml, P-2771MP, Invitrogen) for 1 h at 4 °C. Finally, the cells were washed 3 times with 1× PBS and resuspended in 1× PBS containing 3% bovine serum albumin and immunofluorescence was detected by flow cytometry (Guava MUSE cell analyzer) using the 532 nm laser line with 576/7 nm dichroic filter. Secondary antibody only control (without primary) verified the absence of nonspecific background staining. Data files were analyzed for mean fluorescence intensity by Flowjo software (Becton Dickson, Ashland, Oregon).

2.2.9 NF-κB and PI3k inhibition

RAW 264.7 macrophages were treated with 15 μM Bay11-7082 (Cayman Chemical), an inhibitor of κB kinase phosphorylation, for 1 h to inhibit the NF-κB signaling pathway, and then cells were treated with LPS (100 ng/mL, 4 h) as described above (Lee et al., 2012). RAW 264.7 macrophages were treated with 25 μM LY294002 (Cayman Chemical), an inhibitor of PI3k activation (Matter et al., 1992), for 1 h, and cells were further treated with LPS (100 ng/mL, 4 h).

Inhibitors were administered prior to LPS treatments; fresh media was applied following inhibition.

2.2.10 Akt ELISA

The commercial kit Akt (phosphor) pSer473 InstantOne ELISA (eBioscience, 85-86042-11) was used to detect activated (phosphorylated) Akt in whole cell lysates. Using 96-well plates, 100μL cell lysates were added to each well and incubated at room temperature for 2 h. Each well was washed 4 times (1 × PBS). Then, 100 μL detection reagent was added to each well for 30 min. Stop solution was added, and absorbance was measured at 450 nm using the Biotek synergy H1 spectrophotometer.

2.2.11 NF-κB ELISA

The commercial kit NF-κB phospho-p65 InstantOne ELISA (eBioscience, 85-86083-11) was used to detect phosphorylated NF-κB in whole cell lysates following the manufacturer's instructions. Briefly, using 96-well plates, 50 μL cell lysates were mixed with 50 μL capture antibody cocktail and incubated at room temperature for 1 h. Each well was washed 3 times (1 × PBS). Then, 100 μL detection reagent was added to each well for 30 min. Stop solution was added, and absorbance was measured at 450 nm using the Biotek synergy H1 spectrophotometer.

2.2.12 Statistical Analysis

Western blots were performed from three or more separate cell cultures (n=3). ELISA analysis was done in two replicate reactions and performed with at least three separate cell cultures (n = 3). The glutathione assay was done in two replicate reactions and repeated with three separate cell cultures (n = 3). The real-time PCR analysis of all samples were done in three replicate reactions for each of three independent cell cultures (n = 3). Flow cytometry of Glut1 surface expression was done in three replicate reactions from three separate cell cultures (n=3). Normality

for each experimental group was assessed by unpaired Shapiro-Wilk test. Significant differences between experimental groups were determined using unpaired Student's t-test comparing two groups and One-way ANOVA and Tukey's *post hoc* analysis were used to compare more than two groups using GraphPad Prism 9 software (GraphPad Software, Inc., La Jolla, CA). Data are expressed as mean test ± SD (mean control ± SD). $p \leq 0.05$ was considered significant.

2.3. Results

2.3.1 LPS induces Glut1 expression in 264.7 macrophages

Due to the potential crosstalk of H_2S with NF-κB activation, we investigated whether H_2S plays a role in NF-κB-mediated Glut1 expression. RAW264.7 macrophages were treated with LPS, and Glut1 protein and mRNA expression was measured. The expression of Glut1 mRNA is significantly increased by 2.4-fold from 0.56 ± 0.15 to 1.70 ± 0.18 ($p<0.001$) by LPS treatment compared with the control (Fig 2.1 **a**). Western blot analysis also confirms that Glut1 protein expression significantly increases by 2.16-fold ($p<0.001$) following treatment with LPS (Fig 2.1 **b**,**c**). These observations show that Glut1 is upregulated upon LPS-induced inflammation and is potentially involved in glucose uptake and metabolism during macrophage inflammation.

2.3.2 NF-κB and PI3k regulate Glut1 expression during LPS stimulation

The IKK complex activates NF-κB by phosphorylating and degrading the inhibitory protein IκB in response to signals such as inflammation. To investigate if NF-κB activation is associated with LPS-induced Glut1 mRNA expression, we treated macrophages with an irreversible IKK kinase inhibitor (Bay11-7042) and analyzed its impacts on LPS-induced Glut1 mRNA expression. ELISA analysis demonstrated that upon treatment with LPS, the protein level of phospho-p65 (NF-κB subunit) increased 2.94-fold (from 0.37 ± 0.02 to 1.09 ± 0.06; $p<0.001$) (Fig 2.2 **a**). Here, our ELISA analysis demonstrates that LPS induces the protein level of

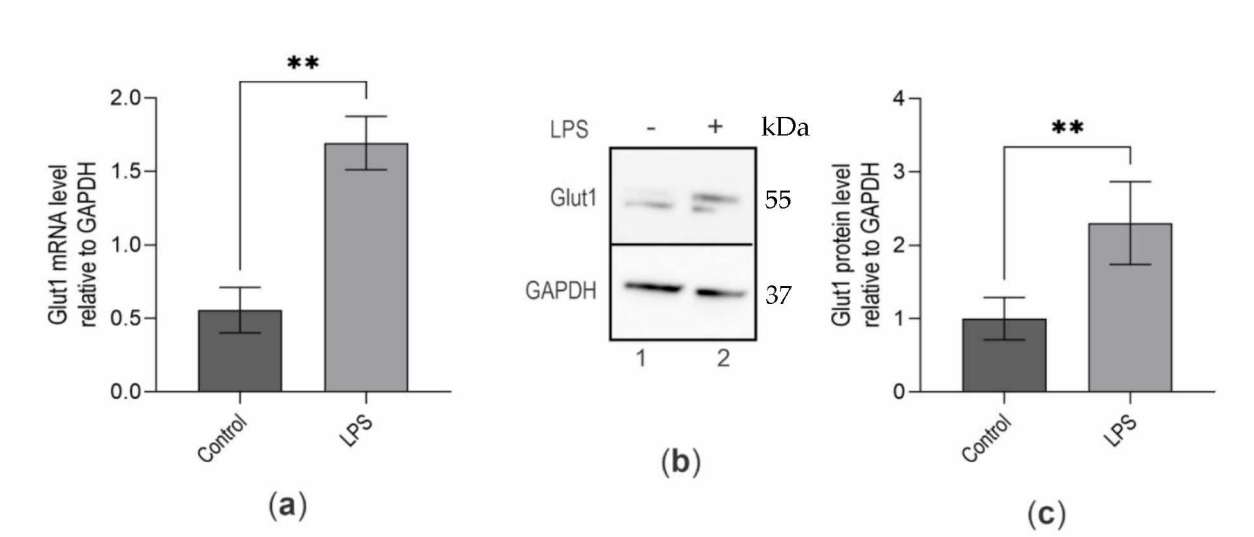

Figure 2.1: LPS induces Glut1 expression in macrophages

RAW264.7 cells were treated with LPS (100 ng/mL) for 4 h, total RNA was isolated, reverse transcribed to cDNA, and RT-qPCR was used to measure the mRNA expression of Glut1. GAPDH was used as a control. The expression (relative to GAPDH) of Glut1 mRNA is shown in panel (**a**). Protein was extracted from macrophages and analyzed by Western blot using antibodies against Glut1 and GAPDH (loading control) (**b**). Quantifications (using ImageJ software) are shown in panel (**c**). Each experiment was repeated at least three times with three replicates each. Data represent mean ± SD (n = 4 **a-b**; n = 3 **c**); **$p < 0.001$.

phospho-Akt (Akt Ser473 subunit) significantly 1.4-fold (from 0.63 ± 0.05 to 0.88 ± 0.09; $p<0.0252$) (Fig 2.2 **b**). To determine if PI3K pathway activation is associated with increased LPS-induced Glut1 mRNA expression, we treated cells with an inhibitor of PI3K (LY294002) and analyzed its effect on LPS-induced Glut1 expression. The LPS-induced increase in Glut1 mRNA expression is significantly decreased by treatment with Bay11-7042 (from 1.70 ± 0.18 to 0.89 ± 0.21; $p<0.011$) (Fig 2.2 **c**). In addition, LY294002 significantly decreased LPS-induce Glut1 mRNA expression (from 1.70 ± 0.18 to 0.75 ± 0.18; $p<0.001$) (Fig 2.2 **c**). Thus, intact PI3k signaling is necessary for LPS-induced Glut1 mRNA expression. These observations suggest that LPS treatment resulted in NF-κB and Akt activation and subsequent Glut1 mRNA expression; inhibiting these pathways reduced Glut1 mRNA expression.

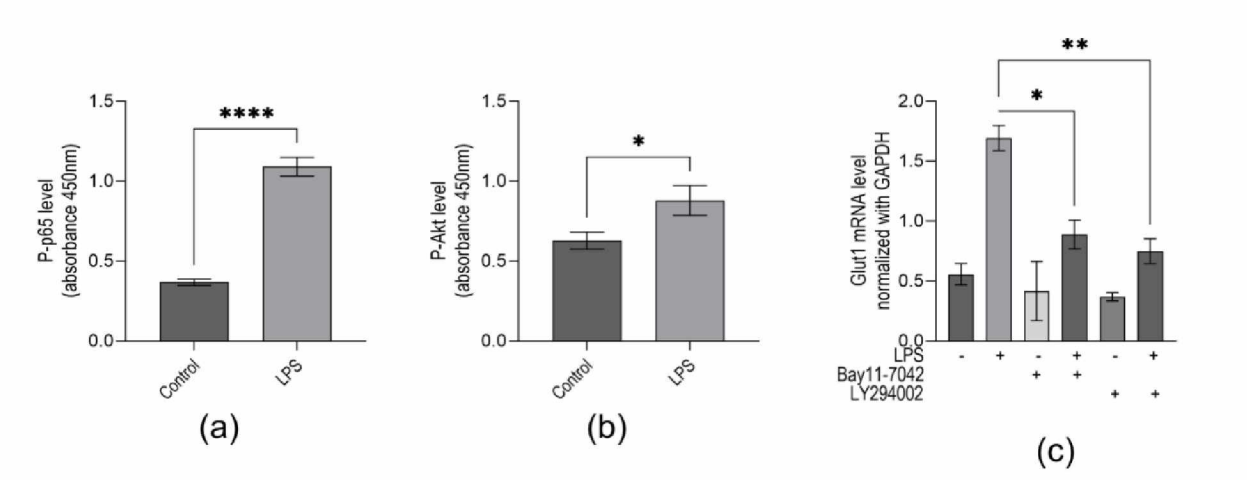

Figure 2.2: LPS activates NF-κB (Phospho-p65) and Akt (ser473), and pharmacological inhibition of NF-κB or PI3k decreases Glut1 expression in macrophages

RAW264.7 cells were initially treated with IKK-inhibitor Bay11-7042 (15 μM) or PI3k inhibitor LY7294002 (25 μM) for 1 h and then treated with LPS for an additional 4 h for RNA analysis. ELISA analyzed proteins by targeting Phosphor-p65 (NF-κB subunit) (panel **a**). Akt protein levels were analyzed by ELISA targeting Phospho-Ser473 (Akt subunit) (panel **b**). RNA was isolated from the above treated and control cells (panel **c**). RT-qPCR was used to measure expression (relative to GAPDH) of Glut1. GAPDH was used as a control. Data represent mean ± SD (n = 4 **a**; n = 3 **b**; n = 3 **c**); *p < 0.05, **p < 0.001, ****p < 0.00001.

CSE regulates LPS-induced Glut1 expression

Here, to investigate if CSE plays any role in regulating LPS-induced Glut1 expression, which impacts glucose uptake, and metabolism, we knocked down CSE (using siRNA) in macrophages, then treated cells with LPS and analyzed its impacts on Glut1 mRNA and protein expression. The expression of CSE is significantly decreased at the protein (from 1 ± 0.25 to 0.12 ± 0.05; p=0.004) and mRNA (from 1.70 ± 0.28 to 0.55±0.02; p=0.002) levels upon application of CSE siRNA (Fig 2.3 **a—c**). LPS-induced Glut1 protein level is significantly decreased upon CSE-knockdown from 2.16 ± 0.53 to 1.22 ± 0.39 (p=0.006) (CSE-siRNA treatment, Fig 2.3 **d**, **e**). LPS-induced Glut1 expression (mRNA level) is significantly decreased upon CSE-knockdown from 1.71 ± 0.28 to 0.55 ± 0.02 (p=0.002) (Fig 2.3 **f**). Phosphorylated (activated) p65 subunit of the NF-κB (NF-κB Phospho-p65) is the active form of NF-κB and

may freely translocate to the nucleus to increase the gene expression of pro-inflammatory target genes. ELISA analysis also showed that LPS-induced Phospho-p65 levels are reduced upon CSE knockdown from 1.13 ± 0.06 to 0.37 ± 0.07 ($p<0.001$) (Fig 2.3 **g**). Phosphorylated (Phospho-)s473 refers to the phosphorylation of the serine 473 residue on the Akt subunit, which signifies activation of the PI3k/Akt signaling pathway. Upon CSE knockdown, the Phospho-s473 (Akt Ser473 subunit) level did not change under CSE-knockdown conditions (Fig 2.3 **h**).

Here, we show that the level of GSH was significantly decreased following LPS treatment from 5.19 ± 0.08 to 4.11 ± 0.62 ($p=0.039$). However, CSE-knockdown significantly decreased GSH levels below the scramble siRNA group in LPS-treated cells from 3.56 ± 0.05 to 2.60 ± 0.02 μM GSH per mg protein ($p<0.001$) (Fig 2.3 **i**). These results demonstrate the important role of CSE and H_2S in regulating GSH levels in macrophages.

2.3.4 H_2S regulates LPS-induced Glut1 expression

To investigate if H_2S plays a role in LPS-induced Glut1 expression, we treated LPS-stimulated macrophages with either 10, 100, or 500 μM GYY4137 for 4h along with LPS; these concentrations achieve a steady state concentration of around 0.32, 3.2, and 16 μmol/L of H_2S for an over 24-hour period, respectively (Lohninger et al., 2015). ELISA analysis showed that in LPS-treated macrophages also treated with 500 μM GYY4137, the Phospho-p65 level was significantly decreased compared to the 0 μM group (from 1.09 ± 0.06 to 0.68 ± 0.22; $p<0.001$) (Fig 2.4 **a**). Interestingly, all tested concentrations of GYY4137 significantly increased Phospho-Ser473 (Akt subunit) levels compared with LPS alone (10 μM – 1.19 ± 0.06; 100 μM – 1.27 ± 0.01; 500 μM – 1.42 ± 0.09 vs. 0.87 ± 0.06; $p<0.001$) (Fig 2.4 **b**). Our RT-qPCR analysis

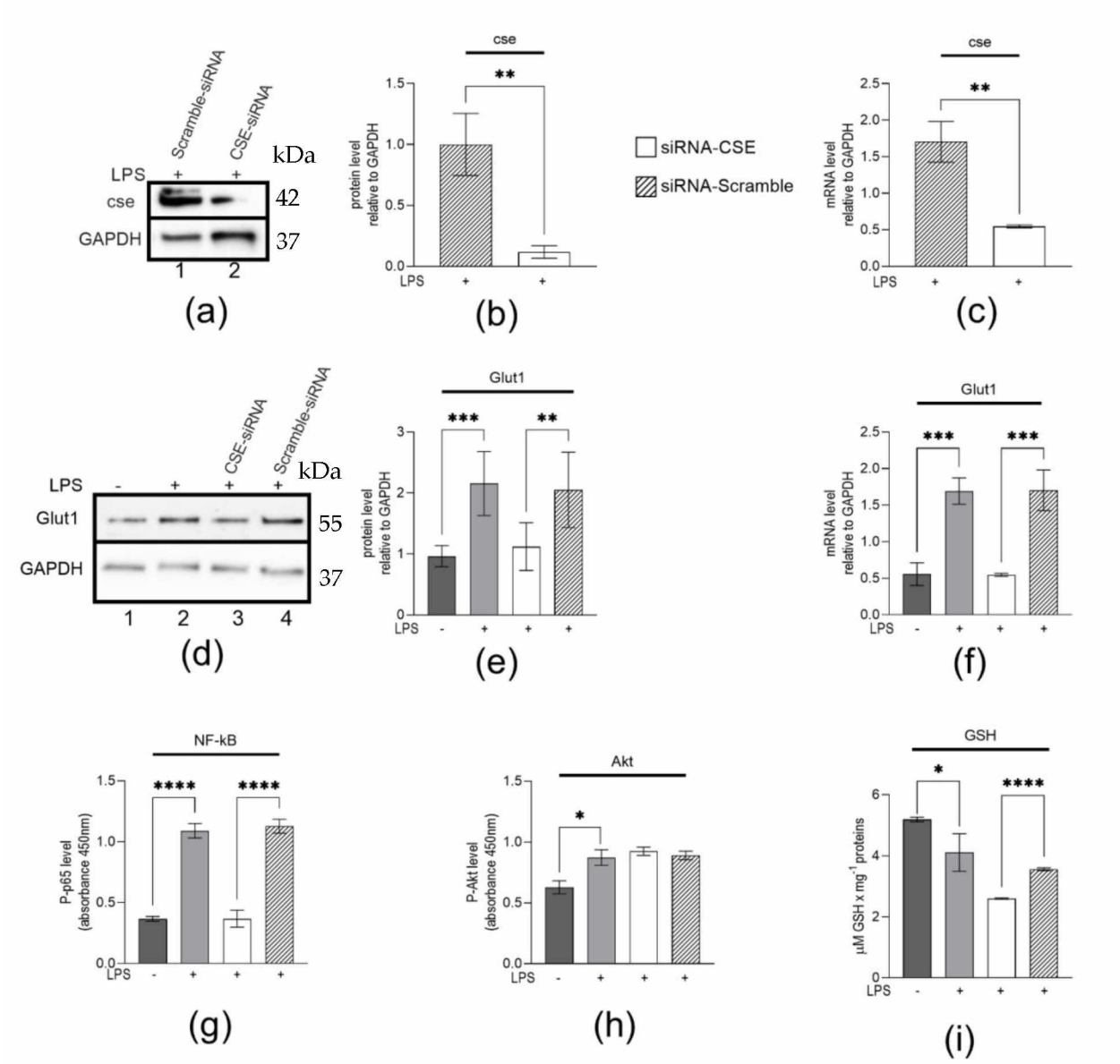

Figure 2.3: Macrophages silenced of CSE decreased NF-κB activation (Phospho-p65), Glut1 expression, and glutathione (GSH) level

RAW264.7 cells were transfected (24 h) with CSE-siRNA or Scramble-siRNA, followed by treatment with LPS. RAW264.7 cells were analyzed by Western blotting using antibodies against CSE, Glut1, and GAPDH (loading control) (panels **a** and **d**). The changes in amounts of Glut1 have been quantified by ImageJ software and shown in panels (**b** and **e**). (Panel **c** and **f**) RT-qPCR was used to analyze the mRNA expression of CSE and Glut1. GAPDH was used as a control. RAW 264.7 cells were analyzed by ELISA targeting Phospho-p65 (NF-κB subunit) (panel **g**) or Phospho-Ser473 (Akt subunit) (panel **h**). GSH levels were determined by using a fluorometric kit. Samples were mixed with reagent and fluorescence intensity was monitored at EX/EM of 490/520 nm. GSH was calculated from the standard curve and reported as GSH * mg

protein^{-1}. Data represent mean ± SD (n = 3 **a-c**; n = 4 **d-e**; n = 3 **f**; n = 4 **g**, n = 3 **h-i**); *p < 0.05, **p < 0.001, ***p < 0.0001, ****p < 0.00001.

demonstrated that the expression of Glut1 increased but not significantly (p=0.086) upon treatment with 500 μM GYY4137, without the addition of LPS. The mRNA expression of Glut1 is significantly reduced upon application of IKK-inhibitor, Bay11-7042 (from 0.82 ± 0.13 to 0.22 ± 0.05; p=0.0018), or PI3K inhibitor, LY294002 (from 0.82 ± 0.13 to 0.31 ± 0.13; p=0.008) (Fig 2.4 **c**). LPS-induced Glut1 mRNA is decreased upon simultaneous treatment of LPS with 500 μM GYY4137 (from 1.70 ± 0.18 to 0.74 ± 0.15, p=0.002) (Fig 2.4 **d**). LPS-induced Glut1 protein level is decreased with treatment of 500 μM GYY4137 from 2.17 ± 0.53 to 1.22 ± 0.43 (p<0.0082) (compare lanes 2 and 4) (Fig 2.4. **e**, quantification in 2.4 **f**). Interestingly, Glut1 protein level with the application of siRNA targeting CSE group is similarly decreased compared to scramble siRNA (from 2.06 ± 0.44 to 1.02 ± 0.28; p<0.001) (compare lanes 5, and 6) (Fig 2.4 **e**, quantification in 2.4 **f**).

To understand the function of H_2S in regulating Glut1 expression, the surface expression of Glut1 on macrophages was measured by flow cytometry. We observed that Glut1 surface expression level was low in the control cells (mean fluorescence intensity MFI = 291 ± 13.6) and significantly increased upon LPS stimulation (689 ± 26.3; p<0.001) (Fig 2.4 **g**). Interestingly, and consistent with previous results, upon CSE-knockdown (CSE-siRNA and LPS treatments), the level of LPS-induced Glut1 surface expression was decreased relative to scramble siRNA and LPS (from 732.6 ± 96.1 to 282.7 ± 10.1; p<0.001). The surface level of LPS-induced Glut1 was similarly decreased following treatment with GYY4173 (GYY4137 and LPS treatments; 433.4 ± 14.2; p<0.001). Scramble siRNA has no significant impact on LPS-induced expression of Glut1.

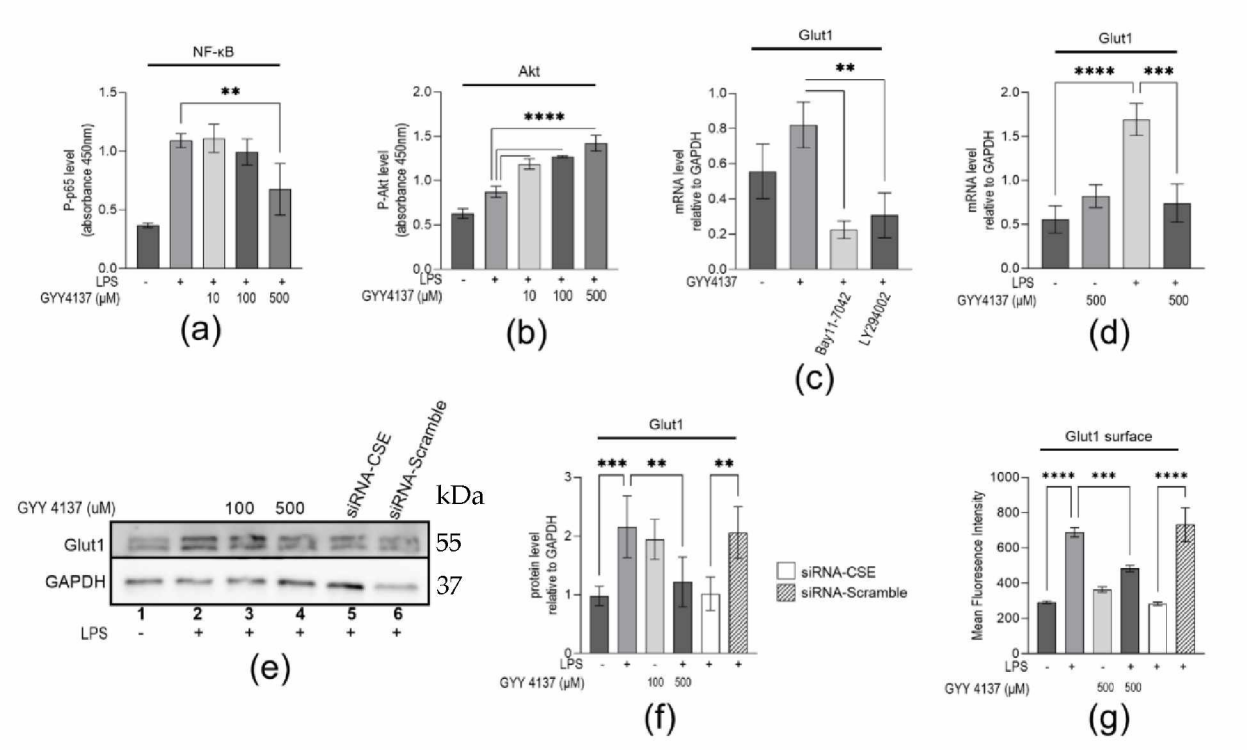

Figure 2.4 High levels of H_2S decreases LPS-induced NF-κB activation and Glut1 expression in macrophages

RAW264.7 cells were treated simultaneously with indicated concentrations of GYY4137 and LPS. (Panel **a** and **b**) RAW 264.7 cells were analyzed by ELISA targeting Phospho-p65 (NF-κB subunit) (panel **a**) or Phospho-Ser473 (Akt subunit) (panel **b**). (**c**) RAW264.7 cells were initially treated with IKK-inhibitor Bay11-7042 (15 μM) or PI3k inhibitor LY7294002 (25 μM) (for 1 h) and then treated with 500 μM GYY4137 for an additional 4 h for RNA. RT-qPCR was used to analyze mRNA for the expression of Glut1 (panel **c**). GAPDH was used as a control. (**d**) RAW264.7 cells were treated with LPS and 500 μM GYY4137 for 4 h for RNA. RT-qPCR analyzed RNA for the expression of Glut1 (panel **d**). GAPDH was used as a control. (**e—f**) RAW264.7 cells were transfected (24 h) with CSE-siRNA or Scramble-siRNA, followed by treatment with GYY4137 and LPS for 4 h. RAW264.7 cells were analyzed by Western blotting using antibodies against Glut1 and GAPDH (loading control) (panel **e**). The changes in amounts of Glut1 have been quantified by ImageJ software and shown in panel (**f**). (**g**) RAW264.7 cells were transfected (24 h) with CSE-siRNA or Scramble-siRNA, followed by treatment with GYY4137 and LPS for 4 h. RAW264.7 cells surface staining was analyzed by flow cytometry using antibodies against Glut1 and secondary antibodies conjugated with R-PE (565/590 nm). Data represent mean ± SD (n = 4 **a**; n = 3 **b-d**; n = 4 **e-f**; n = 3 **g**); **p < 0.001, ***p < 0.0001, ****p < 0.00001.

2.4. Discussion

Activation of the inflammatory response in macrophages is fueled primarily by glucose metabolism (Ganeshan and Chawla, 2014). Due to Glut1's role as the primary rate-limiting glucose transporter in macrophages (Freemerman et al., 2014; Obaid et al., 2021), studying its regulation will elucidate pathways that influence inflammatory responses. Evidence that H_2S regulates the expression of Glut1 and decreases its pro-inflammatory effect (Lohninger et al., 2015) suggests the potential role of H_2S and H_2S-producing enzymes in glucose metabolism in macrophages. Thus, the CSE/H_2S system's role in Glut1 metabolism may be a relevant target for controlling macrophage inflammation.

We investigated the CSE/H_2S system in inflammation-induced metabolic reprogramming in macrophages. Our results showed that the endogenous CSE/H_2S system supports NF-κB activation and Glut1 expression under LPS-induced inflammation in macrophages, while H_2S-donor molecules inhibit NF-κB and Glut1 at high levels, suggesting a regulatory network that may influence glucose metabolism and induce metabolic reprogramming in macrophages during inflammation.

We confirmed that Glut1 expression is induced in macrophages upon LPS stimulation, in accordance with previous studies (Freemerman et al., 2014; Obaid et al., 2021). CSE expression in macrophages is well known to play the primary role in H_2S generation during the immune response (Badiei et al., 2014, 2016; Zheng et al., 2013). CSE expression in macrophages highlights its importance in macrophage biological activities.

NF-κB plays a central role in inflammatory signaling by regulating the transcription of many pro-inflammatory cytokines, chemokines, and inflammatory mediators (Tak and Firestein, 2001). We demonstrated that the inhibition of IKK by Bay11-7042 significantly reduces LPS-

induced Glut1 expression, implicating NF-κB in inflammation-induced Glut1 expression. In addition, our results suggest PI3k activity is associated with LPS-induced Glut1 expression. Indeed, inhibition of PI3k activation using the PI3k inhibitor LY294002 reduced LPS-induced Glut1 expression, demonstrating its role in macrophage metabolic function. NF-κB and PI3k/protein kinase B (Akt) signaling pathway, in addition to hypoxia-inducible factor (HIF)-1α, control metabolic reprogramming in macrophages and provoke Glut1 expression (Covarrubias et al., 2015; Freemerman et al., 2014; Lohninger et al., 2015; Ozes et al., 1999). Results from our NF-κB and PI3k inhibition studies agree with previous reports indicating that these systems control macrophage inflammatory metabolism and Glut1 expression (Covarrubias et al., 2015; Freemerman et al., 2014; Ozes et al., 1999). Next, we investigated the role of endogenous and exogenous H_2S in influencing these pathways in macrophages.

We demonstrate that knockout of the CSE gene with siRNA decreased NF-κB activation in response to LPS stimulation, consistent with a previous report (Badiei et al., 2014). Glut1 expression is also decreased in LPS-treated macrophages genetically silenced of CSE, suggesting an impairment of the inflammation-induced metabolic reprogramming. These observations suggest a role for CSE in regulating Glut1 expression via NF-κB.

The dual effects of ROS on NF-κB signaling pose a challenge, as they can either stimulate or inhibit activity, depending on the cellular context and upstream pathways, leading to inconsistencies in the literature (Morgan and Liu, 2010). ROS can inactivate protein tyrosine phosphatases by oxidizing their catalytic cysteine residues and activating kinases and other enzymes (Groen et al., 2005; Morgan and Liu, 2010; Paulsen and Carroll, 2010). The oxidation and inhibition of c-Jun N-terminal kinase (JNK)-inactivating phosphatases lead to sustained JNK activation, resulting in cytochrome c release, caspase 3 cleavage, and necrotic cell death (Kamata

et al., 2005). Initial oxidation of cysteines is reversible, but further oxidation results in irreversible inactivation and S-glutathionylation and requires reduction to regain protein activity. Previously, Sen et al. (2012) reported that H_2S-mediated persulfidation modified cysteine residues of the p65 subunit of NF-κB, which enhanced binding to anti-apoptotic genes (Sen et al., 2012). Our CSE gene knockout studies demonstrate that CSE plays a role in NF-κB activation and that silencing CSE suppresses NF-κB-DNA binding potential, evidenced by the decreased presence of phosphorylated p65; when the p65 subunit of the NF-κB transcription factor is phosphorylated, it becomes activated and can translocate to the nucleus to bind to specific DNA sequences, leading to increased gene expression of pro-inflammatory cytokines and other target genes. These results show the importance of the CSE/H_2S system to support NF-κB activation and the pro-inflammatory function in macrophages. However, additional work is required to elucidate the exact mechanisms that coordinate the interactions between endogenous H_2S produced by CSE and inflammatory NF-κB activity.

Glucose metabolism and signaling involve many upstream regulators, such as PI3k/Akt. In this study, we show that H_2S from the donor molecule, GYY4137, increases the level of activated Akt in LPS-stimulated cells. Previous studies on THP-1 macrophages have found that Akt activation stabilizes HIF-1α to induce Glut1 expression (Hayashi et al., 2004). In lymphoid cell line FL5.12, PI3k activity was reported to regulate Glut1 trafficking and activated Akt was sufficient to maintain glucose uptake and surface Glut1 without cytokine stimulation (Wieman et al., 2007b). Akt activity was also reported to be critical for the resolution of inflammation and induce anti-inflammatory alternatively activated (M2) macrophages (Vergadi et al., 2017). Here, we show that H_2S donor molecules promote Akt activity and potentially serve an anti-

inflammatory role. Indeed, H_2S was previously shown to promote the resolution of inflammation and injury during colitis via Akt activity (Flannigan et al., 2015).

We show that the role of the CSE/H_2S system is implicated in the expression of Glut1 under LPS stimulation via NF κB; we next investigated whether the H_2S donor molecule, GYY4137, can alter these same systems and Glut1 expression. We show that NF-κB activation is decreased in macrophages upon treatment with 500 μM GYY4137 under LPS stimulation. 10 μM and 100 μM GYY4137 had little effect on NF-κB activation and Glut1 expression. In addition, we show that treatment with 500 μM GYY4137 decreased LPS-induced Glut1 expression. Our results show that high levels of H_2S decrease NF-κB activation, potentially attenuating the macrophage pro-inflammatory response. Our results support the conclusion that H_2S donors may serve an anti-inflammatory function due to the attenuation of NF- κB activation.

Though H_2S has been considered a tissue protectant in some pathological conditions, the mechanisms of tissue protection are controversial. This role of H_2S may involve its influence upon indirect downstream effects. The controversial roles of H_2S are particularly important in inflammation research. Several groups have previously reported the anti-inflammatory activity exhibited by H_2S donor molecules upon NF-κB activity in macrophages (Lohninger et al., 2015; Whiteman et al., 2010). Overall, we provide evidence that the CSE/H_2S system regulates inflammation and plays a role in LPS-induced Glut1 expression. Future studies will be required to elucidate how the flux through various glycolytic pathways, such as the Pentose Phosphate Pathway, is influenced by H_2S. Indeed, H_2S influence on ROS is suggested in numerous studies (Kimura and Kimura, 2004; P. Li et al., 2021; Sun et al., 2012; Wang et al., 2020; Zhang et al., 2020; Zhu et al., 2022). Here, we demonstrate that endogenous CSE/H_2S are important for GSH production and serve a role in antioxidant defenses. However, absent CSE, GSH levels were

markedly decreased but were not associated with increased NF- κB activation within our study parameters. The role of the CSE/H_2S system upon Glut1 expression suggests the potential role played by endogenous H_2S to support pro-inflammatory flux through ROS-producing glycolytic pathways (Freemerman et al., 2014).

Based on our H_2S-donor experiments, we hypothesize that exogenous H_2S may exert an inhibitory influence on ROS production, inhibiting pro-inflammatory response in macrophages. Additionally, we showed that H_2S donor treatment increased Akt activation; thus, it is suggested that H_2S donors exert an anti-inflammatory role during TLR4 stimulation. It is necessary to investigate the role of these systems in primary macrophages, as this study is limited to the investigation of linear mouse RAW264.7 cell line. Overall, our observations demonstrate the novel regulatory pathway that H_2S influences in macrophage immune response.

2.5. References

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Chapter 3 General Conclusion

Inflammatory diseases are characterized by excessive inflammation, and macrophages are central players in the pathogenesis of these disorders. It is widely accepted that metabolism is a crucial regulator of macrophage activation and inflammation. Recently, the gasomediator H_2S has emerged as a potential regulator of cellular energy metabolism, but its precise mechanisms of action in macrophage inflammatory response are not fully understood.

Glut1 is the predominant glucose transporter in macrophages, and its expression is increased in response to inflammatory activation to facilitate the uptake and utilization of glucose, which is essential for the inflammatory response (Freemerman et al., 2014, 2019). This regulatory network is complex and involves the rapid production of ROS and NO, which act as signaling molecules to activate transcription factors such as HIF-1α, NF-κB, and AP-1 (Freemerman et al., 2014; Li et al., 2018; Meyer et al., 1993). Increased glucose uptake and metabolism in immune cells is crucial for upregulating the production of ROS by the NOX enzyme through the synthesis of NADPH, which is a key cofactor required for NOX-derived ROS generation (Freemerman et al., 2014). During macrophage inflammatory response, NF-κB acts as a transcriptional activator of HIF-1α, inducing the expression of Glut1 to increase glucose uptake and utilization (Rius et al., 2008).

The impact of H_2S on cellular energy metabolism through its regulatory role in mitochondrial respiration has been hypothesized to modulate cellular bioenergetics, but its precise effects on immune cell inflammatory responses remain unclear. While high concentrations of H_2S block cytochrome c oxidase activity (Libiad et al., 2019), low physiologic concentrations facilitate its functioning as an electron transport chain substrate via the enzymatic activity of sulfide quinone oxidoreductase (Kumar et al., 2021; Vitvitsky et al., 2021). In the

event of inhibition of the ETC by excessive H_2S, increased glycolytic flux is triggered and increases cytosolic nicotinamide adenine dinucleotide (NADH) levels (Vitvitsky et al., 2021). To prevent this, lactate production is elevated and acts as a counterbalance, creating a redox neutral cycle that enables the cell to continue generating ATP via glycolysis, despite the inhibition of the ETC. The cycle relies on the functioning of the electrogenic glutamate-aspartate transporter, which plays a vital role in the malate-aspartate shuttle (Vitvitsky et al., 2021). This interaction demonstrates how H_2S may signal to influence cellular energy metabolism, and place increased glucose substrate demands on cells exposed to H_2S.

GYY4137, an H_2S-donor molecule, increased Glut1 expression in THP-1 macrophages by stabilizing HIF-1α under normal oxygen conditions, inducing the transcription of Glut 1 (Lohninger et al., 2015). However, the role of H_2S in pro-inflammatory macrophages is complex and remains unclear. While endogenous upregulation of the H_2S-producing enzyme, CSE, is recognized to support pro-inflammatory response in macrophage (Badiei et al., 2014; 2015), studies that have utilized H_2S donors show that high levels may abrogate inflammation- induced NF-κB in macrophage (Whiteman et al., 2010). The conflicting results underscore the importance of considering the concentration of H_2S and its source in determining its function. Our study aimed to elucidate whether endogenous H_2S can regulate LPS-induced Glut1 expression via NF-κB modulation, to shed light on the role of H_2S in Glut1-mediated glucose metabolism, a crucial aspect of macrophage inflammatory responses.

To study the role of H_2S on inflammation-induced metabolic reprogramming in macrophages, RAW 264.7 cells were silenced of CSE, the major source of H_2S in these cells, and the effect on Glut1 was measured. Knocking out CSE lowered levels of LPS-induced Glut1 expression suggesting that H_2S may be involved in regulating Glut1 expression in response to

immune activation. Additionally, we found that siRNA-mediated knockout of CSE resulted in reduced activation of NF-κB. Mechanistically the cause of these effects is still in the early stages of investigation. However, the dominant hypothesis for H_2S signaling is that its effects are elicited by protein persulfidation, leading to the modification of cysteine residues (Mustafa et al., 2009). Quantitative analysis by other researchers has revealed that protein persulfidation in cells increases when CSE is upregulated or with the administration of H_2S-donor molecules (Gao et al., 2015), and persulfidation of NF-κB has been previously shown to enhance gene transcription activities (Sen et al., 2012). Therefore, this is a future research direction to elucidate the mechanism of our results in this work.

The NF-κB signaling cascade interacts with several parallel pathways, including the signaling cascades initiated by PI3K and Akt to influence glucose metabolism (Kane et al., 1999). Our results have demonstrated that H_2S donor molecule administration enhanced LPS-induced Akt activity. Blockade of either PI3K or NF-κB inhibited LPS-induced Glut1 expression and thus prevented metabolic reprogramming in macrophages. Our findings have shed light on the complex regulatory mechanisms governing Glut1 expression in immune-stimulated macrophages, with implications for metabolic rewiring during inflammation. Administration of low levels of H_2S-donor molecules did not significantly affect Glut1 expression or NF-κB activity. However, administering a high concentration of H_2S-donors reduced NF-κB activation and Glut1 expression. These findings suggest a delicate balance of H_2S-mediated signaling and other regulatory mechanisms in the immune response and necessitate further investigation regarding inflammatory diseases characterized by excessive inflammation.

The crosstalk between immunity and cellular metabolism is partially mediated by redox activity (Kolliniati et al., 2022; Wang et al., 2017). Molecular H_2S and cysteine directly

contribute to antioxidant levels in cells (Ida et al., 2014; Sun et al., 2012) . CSE and H_2S regulate cysteine levels in cells, and cysteine is the limiting substrate for GSH production (Lee et al., 2014). Here, we have demonstrated that silencing CSE expression led to decreased levels of GSH when treated with LPS.

For future studies, it will be necessary to investigate the role of the CSE/H_2S system upon inflammation induced glucose metabolism in primary macrophages, as this study is limited to the investigation of linear mouse RAW264.7 cell line. Therefore, further investigation is necessary to confirm these findings in primary cells. Overall, our findings suggest that H_2S can directly modulate macrophage Glut1 expression, which is a critical regulator of macrophage inflammatory response.

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Appendix: Cystathionine γ-lyase and hydrogen sulfide modulates glucose transporter Glut1 expression via NF-κB and PI3k/Akt in macrophages during inflammation

PLOS ONE

RESEARCH ARTICLE

Cystathionine γ-lyase and hydrogen sulfide modulates glucose transporter Glut1 expression via NF-κB and PI3k/Akt in macrophages during inflammation

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Abstract

Macrophages play a crucial role in inflammation, a defense mechanism of the innate immune system. Metabolic function powered by glucose transporter isoform 1 (Glut1) is necessary for macrophage activity during inflammation. The present study investigated the roles of cystathionine-γ-lyase (CSE) and its byproduct, hydrogen sulfide (H_2S), in macrophage glucose metabolism to explore the mechanism by which H_2S acts as an inflammatory regulator in lipopolysaccharide- (LPS) induced macrophages. Our results demonstrated that LPS-treated macrophages increased Glut1 expression. LPS-induced Glut1 expression is regulated via nuclear factor (NF)-κB activation and is associated with phosphatidylinositol-3-kinase PI3k activation. Small interfering (si) RNA-mediated silencing of CSE decreased the LPS-induced NF-κB activation and Glut1 expression, suggesting a role for H_2S in metabolic function in macrophages during pro-inflammatory response. Confoundingly, treatment with GYY4137, an H_2S-donor molecule, also displayed inhibitory effects upon LPS-induced NF-κB activation and Glut1 expression. Moreover, GYY4137 treatment increased Akt activation, suggesting a role in promoting resolution of inflammation. Our study provides evidence that the source of H_2S, either endogenous (via CSE) or exogenous (via GYY4137), supports or inhibits the LPS-induced NF-κB activity and Glut1 expression, respectively. Therefore, H_2S may influence metabolic programming in immune cells to alter glucose substrate availability that impacts the immune response.

Introduction

Hydrogen sulfide (H_2S) is an endogenously produced inflammatory mediator increasingly recognized for its role in various inflammatory diseases. H_2S functions as a signaling molecule at physiologic levels influencing several biological processes [1–4]. H_2S is produced in mammalian cells from L-cysteine predominately by cystathionine γ-lyase (CSE), cystathionine beta-

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synthase (CBS), and 3-mercaptopyruvate sulfotransferase (3-MST) [5]. H_2S is a pro-inflammatory mediator produced by the activity of CSE in macrophages [6, 7], which supports their further activation [8]. Excessive H_2S is proposed to promote inflammation and tissue damage in an animal model of sepsis [9, 10], and inherited retinal disease [11]. A recent report demonstrates the correlation of higher concentrations of plasma levels of H_2S with an early inflammatory response in septic patients, suggesting that the early elevated H_2S concentrations influence substance P levels [12]. Thus, the associated role of H_2S in inflammatory disease necessitates the elucidation of its role in regulating inflammation in macrophages.

Immune responses triggered by Toll-like receptor (TLR) activity in macrophages stimulate the upregulation of CSE and concomitant production of H_2S, which requires nuclear factor kappa-light-chain enhancer of B-cells (NF-κB), p38 mitogen-activated protein kinase (MAPK), and extracellular signal-regulated kinase (ERK) signaling pathways [7, 13]. NF-κB is a well-characterized signaling network for regulating inflammation [14]. It has been reported that the NF-κB and PI3k/protein kinase B (Akt) signaling pathway control metabolic function via the regulation of hypoxia-inducible factor (HIF)1α in macrophages [15–18]. Glucose transporter isoform 1 (Glut1) was induced by H_2S activity to stabilize HIF1α in an *in vitro* study of human macrophages [16], suggesting a role for H_2S in glucose metabolic function.

Immunity and cellular metabolism are two fundamentally linked systems, and the crosstalk between them regulates the immune function of cells [19]. The glucose transporter (Glut) family is a class of hexose transporters that imports glucose into cells. The most widely expressed glucose transporter in tissue and cells is Glut1—which maintains glucose levels to support cellular energy requirements [20, 21]. Glut1 is the primary rate-limiting glucose transporter in macrophages and a critical regulator of macrophage inflammatory response [18]. Upon macrophage stimulation, Glut1 levels are increased via NF-κB [18] which requires PI3K/Akt activation as well [15]; These pathways then culminate to stabilize HIf1α, which in turn regulates Glut1 expression to induce glycolysis for inflammatory activation [22]. The rapid increase in Glut1 during acute inflammation in macrophages drives increased glucose uptake and utilization to induce a reactive oxygen species (ROS) driven pro-inflammatory response [18].

In this study, we investigated the CSE/H_2S system's role in macrophage metabolic function during the immune response and examined the role of NF-κB and Akt in this process. We hypothesized that CSE/H_2S system plays a critical role in inflammation-induced glucose metabolism due to previous evidence that crosstalk exists between H_2S and the inflammatory NF-κB activities [8]. Our study provides evidence that this system regulates the expression of Glut1 and regulates glucose metabolism in macrophages to influence the immune response. Our study provides evidence that endogenous CSE-derived H_2S supports the pro-inflammatory expression of Glut1. We also show that exogenous H_2S from H_2S-donor molecules reduced LPS-induced Glut1.

Materials and methods

Macrophage cell culture

RAW264.7 (ATCC, Manassas, VA) murine macrophage cell lines were cultured in DMEM (Gibco, Waltham, MA) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS; Cell Applications Inc., San Diego, CA), 100 units/ml penicillin, and 100 μg/ml streptomycin (Gibco) and maintained at 37˚C in a humidified atmosphere containing 5% CO_2. Cells were counted to seed 2.4 x 10^6 cells on 6-well culture plate and grown to confluence. After reaching 70% confluence, macrophages were ready for treatment.

Macrophage treatment with lipopolysaccharide (LPS)

Macrophages were stimulated with *E. coli*-derived LPS (100ng/mL; Invitrogen, Waltham, MA) for 4 h. The concentration of LPS has been reported by other laboratories to induce immune and pro-inflammatory responses in macrophages [7, 8, 13, 23]. After treatment, macrophages were harvested for the preparation of RNA and protein analysis.

Protein extraction and Western blot

The treated macrophage cells were washed with ice-cold PBS and then lysed in RIPA cell lysis buffer, and 1% Halt protease inhibitor cocktail (Thermo Fisher). The resulting cell lysates were centrifuged for 20 min. at 20,000 g at 4˚C, and the protein concentrations in the supernatants were determined using a Pierce BCA protein assay kit (Thermo Fisher) [24]. 20 μg proteins were loaded onto 10% SDS-PAGE gels, followed by electro-transfer onto nitrocellulose-membrane (Bio-Rad). The membranes were blocked in 1 × TBST (0.1% Tween-20, 20 mM Tris–Cl (pH 8.0), and 150 mM NaCl) containing 5% nonfat dry milk powder and then incubated with the primary antibodies against Glut1 (1:1000 dilution, 66290, Proteintech), GAPDH (1:1000 dilution, 60004, Proteintech), overnight at 4˚C. Membranes were washed 3 times (1xTBST), and incubated with horseradish peroxidase-conjugated secondary antibodies (1:10000, SA00001, Proteintech) for 1 h at room temperature and then washed 3 times (1 × TBST). Lastly, immunoreactive proteins were detected using enhanced chemiluminescence detection kit (Bio-Rad). The band density was quantified by Image J 1.8.0172 software (National Institutes of Health) and the representative data were experiment normalized to nontreated control.

RNA extraction and RT-qPCR

Total RNA from cells was extracted using Trizol and chloroform reagents (Invitrogen) following the manufacturer's instructions. Sample concentrations were determined using Nanodrop One. First-strand cDNA synthesis was performed on 5μg total RNA using M-MLV reverse transcriptase (Invitrogen) and random hexamers (IDT, Coralville, IA) and stored at −20˚C. PowerUp SYBR Green Mix (Applied Biosystems, Waltham, MA) was used according to the manufacturer's instructions in a 384-well format. To compare the mRNA levels between different samples, the $2^{-\Delta Ct}$ [25] method was used; and data were normalized to GAPDH. Experiments were run in triplicate; each sample represents three technical repeats. The sense and antisense primers were designed using the PrimerQuest Tool from Integrated DNA Technologies (Table 1).

siRNA-mediated knockdown of CSE gene

Silencer Select pre-designed siRNAs (Ambion, Austin, TX) targeting the CSE gene and negative scramble control siRNA were used in the gene silencing experiments. CSE is the dominant H_2S-producing enzyme in several tissues, including macrophage [4, 26]; silencing CSE diminishes macrophage capacity to produce endogenous H_2S. Following manufacturer instructions

Table 1. PCR primer sequences.

Gene	Forward (5′–3′)	Reverse (5′–3′)
Glut1	GATCTGAGCTACGGGGTCTT	TGTAGAACTCCTCAATAACCTTCTG
CSE	CAAAGCAACACCTCGCACTC	ATGCAAAGGCCAAACTGTGC
GAPDH	CGTCCCGTAGACAAAATGGT	GAGGTCAATGAAGGGGTC

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for lipofectamine RNAiMAX (Invitrogen), cells were incubated with 5pmol siRNA-lipofectamine complex for 24 h. After incubation, the medium was replaced, and cells were further treated.

NF-κB activity

The commercial kit NF-κB phospho-p65 InstantOne ELISA (eBioscience, 85-86083-11) was used to detect phosphorylated NF-κB in whole cell lysates following the manufacturer's instructions. Briefly, using 96-well plates, 50μL cell lysates were mixed with 50μL capture antibody cocktail and incubated at room temperature for 1 h. Each well was washed 3 times. Then, 100μL detection reagent was added to each well for 30 min. Stop solution was added, and plate readings were performed immediately at 450 nm.

Akt activity

The commercial kit Akt (phosphor) pSer473 InstantOne ELISA (eBioscience, 85-86042-11) was used to detect activated (phosphorylated) Akt in whole cell lysates. Using 96-well plates, 100μL cell lysates were added to each well and incubated at room temperature for 2 h. Each well was washed 4 times. Then, 100μL detection reagent was added to each well for 30 min. Stop solution was added, and plate readings were performed immediately at 450 nm. Plate readings were performed at 450 nm. Akt activity was measured following 1 h treatments.

NF-κB inhibition and PI3K inhibition

RAW 264.7 macrophages were treated with 15μM Bay11-7082 (Cayman Chemical), an inhibitor of κB kinase phosphorylation, for 1 h to inhibit the NF-κB signaling pathway, and then cells were further treated [27]. RAW 264.7 macrophages were treated with 25μM LY294002 (Cayman Chemical), an inhibitor of PI3K activation [28], for 1 h, and cells were further treated. Inhibitors were administered prior to LPS and GYY4137 treatments; fresh media was applied following inhibition.

Flow cytometry to measure Glut1 surface levels

For immunofluorescence surface staining of macrophages, cells were suspended and fixed at a concentration of 10^6 cells per 100μL in 4% paraformaldehyde (PFA) for 15 min at room temperature. The cells were then washed with 1X PBS (3 times) and suspended (10^6 cells per 100μL) in antibody dilution buffer (1X PBS containing 3% bovine serum albumin) with primary antibodies targeting Glut1 for 1 h at 4˚C. After that the cells were washed 3 times with 1X PBS followed by incubation with R-phycoerythrin R-PE (Invitrogen), conjugated secondary antibody for 1 h at 4˚C. Finally, the cells were washed 3 times with 1X PBS and resuspended in 1X PBS containing 3% bovine serum albumin and read by flow cytometry (Guava MUSE cell analyzer). Data files were analyzed for mean fluorescence intensity by floreada.io software.

Glutathione level assay

GSH level in macrophages was determined using a one-step fluorometric kit (Fluorometric-Green, ab138881, Abcam) according to the manufacturer's protocol. Potassium phosphate EDTA buffer (KPE) was prepared freshly, immediately prior to experiments [29]. Following treatments, macrophages were removed from culture plates and counted to 10^6 cells per aliquot. Aliquoted cells were lysed in 0.5mL KPE buffer containing 0.1% Triton X-100 & 0.6% sulfosalicylic acid and kept on ice. Cell lysates were then vortexed for 15 seconds and

centrifuged at 8,000 g for 10 minutes. The supernatants were transferred to pre-chilled micro-centrifuge tubes. The concentration of total protein in each sample was determined by Pierce BCA protein assay kit. Samples were then mixed 1:1 with glutathione detection reagent to a final volume of 100μL on 96-well plates and incubated in the dark at room temperature for 30 min. Then, fluorescence intensity was monitored at EX/EM of 490/520 nm. GSH was calculated from the standard curve and reported as GSH * mg protein^{-1}.

Statistical analysis

Statistical differences between experimental groups were determined using statistics software within GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). For all experiments, results are reported for at least n = 3. Data are expressed as mean + SD. Statistical significance was determined for groups of 3 or more by 1-way analysis of variance (ANOVA) and Tukey's multiple comparisons. For groups of 2, statistical significance was determined by unpaired Student's t-test. $P \leq 0.05$ was considered statistically significant.

Results

LPS induces Glut1 expression in 264.7 macrophages

Studies have demonstrated that H_2S plays roles in inflammation and elicited a biphasic effect on pro-inflammatory NF-κB activation depending on its source and concentration [8, 23]. NF-κB gene regulatory activities increase Glut1 gene transcription in macrophages during LPS stimulation to fuel the inflammatory response [18, 30]. Due to the potential crosstalk of H_2S with NF-κB activation, we investigated whether H_2S plays any role in NF-κB-mediated Glut1 expression. RAW264.7 macrophages were treated with LPS, and Glut1 expression was measured. Briefly, RNA from the control and LPS-treated macrophages were reverse-transcribed into cDNA and analyzed by qPCR using primers specific to Glut1. The expression of Glut1 is significantly increased by 2.4-fold (1.70±0.18 vs. 0.56±0.15; $p<0.001$) by LPS treatment vs. control (Fig 1a). Western blot analysis also confirms that Glut1 protein expression significantly increases by 2.16-fold ($p<0.001$) following treatment with LPS (Fig 1b, quantification in Fig 1c). These observations show that Glut1 is upregulated upon LPS-induced inflammation and is potentially involved in glucose uptake and metabolism during inflammation in macrophages.

NF-κB and PI3k regulate glut1 expression during LPS stimulation

The transcription factor NF-κB activation plays a central role in inflammation and immune response. Without inflammatory stimulus, NF-κB is complexed with IκBα and remains inactive. LPS interaction with TLR-4 stimulates a phosphorylation cascade leading to IκBα degradation and NF-κB translocation to the nucleus, activating the transcription of target genes [31]. To investigate if NF-κB activation is associated with LPS-induced Glut1 expression, we treated macrophages with an irreversible IKK kinase inhibitor (Bay11-7042) and analyzed its impacts on LPS-induced Glut1 expression. ELISA analysis demonstrates that upon treatment with LPS, the level of phospho-p65 (NF-κB subunit) is increased 2.94-fold (0.37±0.02 vs. 1.09 ±0.06; $p<0.001$) (Fig 2a). Studies have demonstrated that inflammatory signaling promotes glucose uptake via PI3k/Akt regulation of Glut1 activity and trafficking [32, 33]. Here, our ELISA analysis demonstrates that LPS induces phospho-Akt (Akt Ser473 subunit) significantly 1.4-fold (0.63±0.05 vs. 0.88 SD±0.09; $p<0.0252$) (Fig 2b). To determine if PI3K pathway activation is associated with increased LPS-induced Glut1 expression, we treated cells with an inhibitor of PI3K (LY294002) and analyzed its effect on LPS-induced Glut1 expression. The

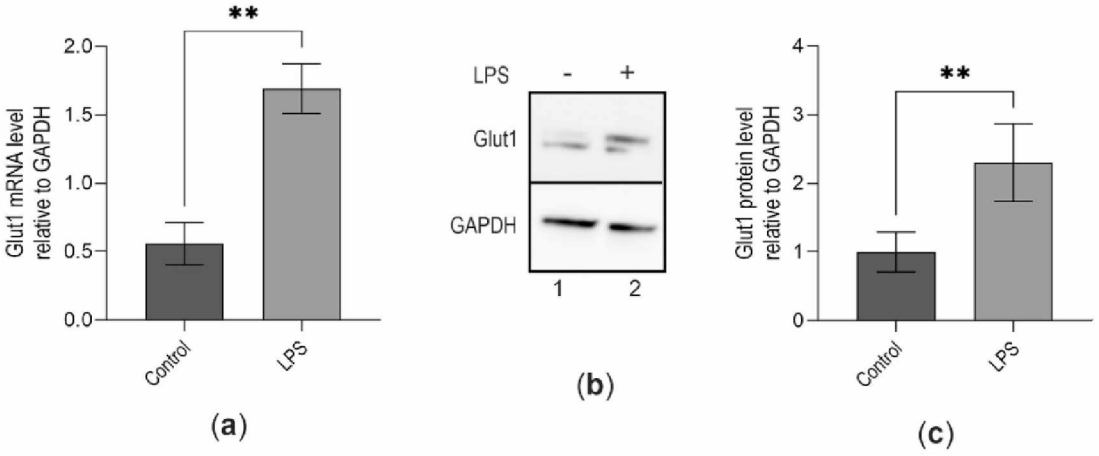

Fig 1. LPS induces Glut1 expression in macrophages. (a–c) RAW264.7 cells were treated with LPS (100 ng/mL) for 4 h, total RNA was isolated, reverse transcribed to cDNA, and RT-qPCR analyzed the expression Glut1. GAPDH was used as a control. The expression (relative to GAPDH) of Glut1 mRNA is shown in panel (**a**). Protein was extracted from macrophages and analyzed by Western blot using antibodies against Glut1 and GAPDH (loading control) (**b**). Quantifications (using ImageJ software) are shown in panel (**c**). Each experiment was repeated at least with three parallel replicates. Data represent mean + SD (n = 3); $^{**}p < 0.001$, $^{***}p < 0.0001$.

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LPS-induced increase in Glut1 expression is significantly decreased by treatment with Bay11-7042 (0.89±0.21 vs. 1.70±0.18; p<0.011). In addition, LY294002 significantly decreased LPS-induce Glut1 expression (0.75±0.18 vs. 1.70±0.18; p<0.001) (Fig 2c). Thus intact PI3k signaling is necessary for LPS-induced Glut1 expression. These observations suggest that LPS treatment resulted in NF-κB and Akt activation and subsequent Glut1 expression; inhibiting these pathways reduced this expression.

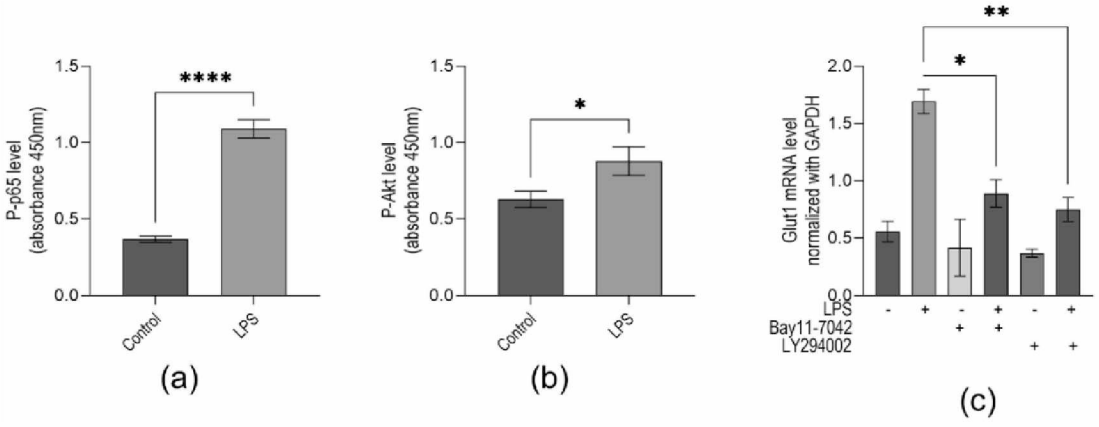

Fig 2. LPS induces phosphorylation of NF-κB subunit p65 and phosphorylation of Akt subunit ser473, and NF-κB and PI3k are associated with Glut1 expression in macrophages. (a–c) RAW264.7 cells were initially treated with IKK-inhibitor Bay11-7042 (15μM) or PI3k inhibitor LY7294002 (25μM) for 1 h and then treated with LPS for an additional 4 h for RNA analysis. ELISA analyzed proteins by targeting phospho-p65 (NF-κB subunit) (panel **a**). Akt proteins were analyzed by ELISA targeting Phospho-Ser473 (Akt subunit) (panel **b**). (**c**) RNA was isolated from the above treated and control cells. RT-qPCR measured expressions (relative to GAPDH) of Glut1. GAPDH was used as a control. Data represent mean ± SD (n = 3); $^{*}p < 0.05$, $^{**}p < 0.001$, $^{****}p < 0.00001$.

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CSE regulates LPS-induced Glut1 expression

It was reported that CSE plays a key role in NF-κB activation [8, 13]. Here, to investigate if CSE plays any role in regulating LPS-induced Glut1 expression, glucose uptake, and metabolism, we knocked down CSE (using siRNA) in macrophages, then treated them with LPS, and analyzed its impacts on Glut1 RNA and protein expression. Briefly, RAW264.7 macrophages were transfected with CSE or scramble siRNAs (24 h) and treated with LPS (4 h). RNA and proteins were isolated and analyzed by RT-qPCR and Western blot. The expression of CSE is significantly decreased at the protein (0.12±0.05 vs. 1±0.25; p = 0.004) and mRNA (0.55±0.02 vs. 1.70±0.28; p = 0.002) levels upon application of CSE siRNA (Fig 3a–3c). Western blot analysis showed that LPS-induced Glut1 protein level is significantly decreased upon CSE-knockdown (1.22±0.39 vs. 2.16±0.53; p = 0.006) (CSE-siRNA treatment, Fig 3d, quantification in Fig 3e). RT-qPCR analysis showed that LPS-induced Glut1 expression (mRNA level) is significantly decreased upon CSE-knockdown (0.55±0.02 vs. 1.71±0.28; p = 0.002) (Fig 3f). ELISA analysis also showed that LPS-induced Phospho-p65 levels are reduced upon CSE knockdown (0.37±0.07 vs. 1.13±0.06; p<0.001) (Fig 3g). However, the Phospho-s473 (Akt Ser473 subunit) level did not change under CSE-knockdown conditions (Fig 3h). Therefore, the CSE/H_2S system supports pro-inflammatory NF-κB activity and glucose metabolism.

Previously, it was shown that inhibition of the CSE/H_2S system dysregulated glutathione (GSH) status, and this could be partially ameliorated with H_2S donor molecules [34]. Here, we show that the level of GSH was significantly decreased following LPS treatment (4.11±0.62 vs. 5.19±0.08; p = 0.039). However, consistent with previous reports, CSE-knockdown conditions significantly decreased GSH levels below the scramble siRNA group in LPS-treated cells (2.60 ±0.02 vs. 3.56±0.05 μM GSH per mg protein; p<0.001) (Fig 3i). These results demonstrate the important role of CSE and H_2S in regulating GSH levels in macrophages.

H_2S regulates LPS-induced Glut1 expression

Previously, Lohninger *et al.* (2015) reported that treatment with the H_2S-donor molecule, GYY4137, induced the expression of Glut1 by stabilizing HIF1α under normal oxygen conditions in THP-1 macrophages [16]. This same study also reported that high concentrations of H_2S decreased NF-κB activation, which is consistent with another group's report on RAW264.7 macrophages [23]. Contrary to previous reports, we suggested the roles of endogenous H_2S to support pro-inflammatory Glut1 expression within the CSE/H_2S system in the previous section. However, exogenous H_2S regulating inflammation-induced Glut1 expression in macrophages is poorly understood. Here, to investigate if H_2S plays any role in LPS-induced Glut1 expression, we treated LPS-stimulated macrophages with either 10, 100, or 500μM GYY4137 for 4h simultaneous to LPS being administered; these concentrations achieve a steady state concentration of around 0.32, 3.2, and 16 μmol/L of H_2S for an over 24-hour period, respectively [16]. The impacts of H_2S levels on NF-κB activation, Akt activation, Glut1 RNA, and protein expression were measured. Briefly, RAW264.7 macrophages were treated with LPS simultaneous with the indicated concentration of GYY4137 for 4h. Initially, we sought to elucidate the effect of H_2S on key pathways that regulate inflammatory glucose uptake and metabolic function (NF-κB and Akt pathways). ELISA analysis showed that in LPS-treated macrophages also treated with 500μM GYY4137, Phospho-p65 levels were significantly decreased compared to the 0μM group (0.68±0.22 vs. 1.09±0.06; p<0.001) (Fig 4a). Interestingly, all tested concentrations of GYY4137 significantly increased Phospho-Ser473 (Akt subunit) levels vs LPS alone (10μM– 1.19±0.06; 100μM– 1.27±0.01; 500μM– 1.42±0.09 vs. 0.87±0.06; p<0.001) (Fig 4b).

RNA and proteins were isolated and analyzed by RT-qPCR and Western blot, respectively. Our RT-qPCR analysis demonstrated that the expression of Glut1 increased but not

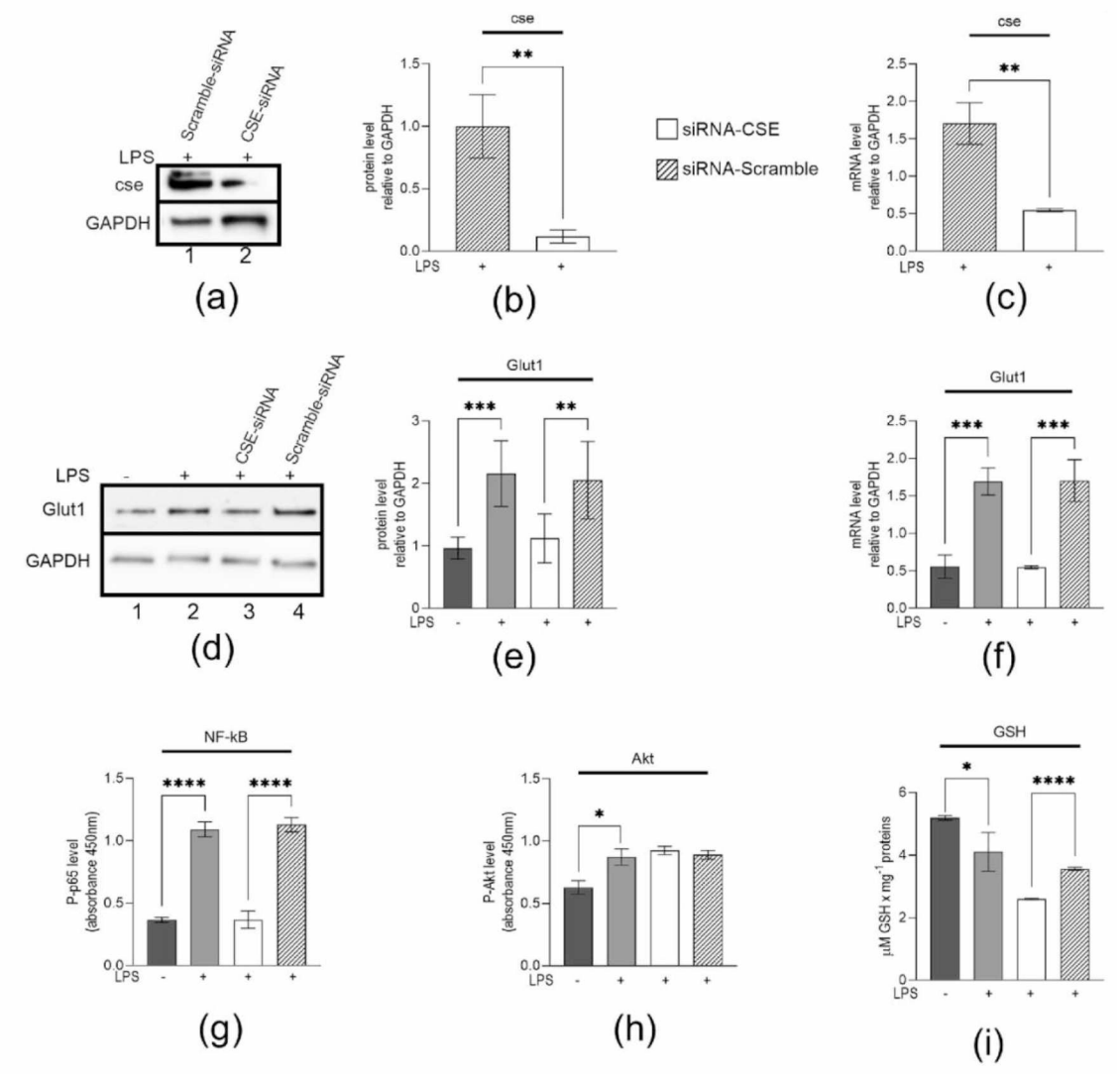

Fig 3. LPS stimulated macrophages genetically silenced of CSE gene to inhibit CSE expression, decreased NF-κB activation (Phospho-p65), Glut1 expression, and glutathione (GSH) level. (a–f) RAW264.7 cells were transfected (24 h) with CSE-siRNA or Scramble-siRNA, followed by treatment with LPS. RAW264.7 cells were analyzed by Western blotting using antibodies against CSE, Glut1, and GAPDH (loading control) (panels **a** and **d**). The changes in amounts of Glut1 have been quantified by ImageJ software and shown in panels (**b** and **e**). (Panel **c** and **f**) RT-qPCR analyzed RNA for the expression of CSE and Glut1. GAPDH was used as a control. RAW 264.7 cells were analyzed by ELISA targeting Phospho-p65 (NF-κB subunit) (panel **g**) or Phospho-Ser473 (Akt subunit) (panel **h**). GSH levels were determined by a fluorometric kit. Samples were mixed with reagent, and fluorescence intensity was monitored at EX/EM of 490/520 nm. GSH was calculated from the standard curve and reported as GSH * mg protein-1. Data represent mean ± SD (n = 3); $^{*}p < 0.05$, $^{**}p < 0.001$, $^{***}p < 0.0001$, $^{****}p < 0.00001$.

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significantly (p = 0.086) upon treatment with 500μM GYY4137, without the addition of LPS. This expression of Glut1 is significantly reduced upon application of IKK-inhibitor, Bay11-7042 (0.22±0.05 vs. 0.82±0.13; p = 0.0018), or PI3K inhibitor, LY294002 (0.31±0.13 vs. 0.82 ±0.13; p = 0.008) (Fig 4c). RT-qPCR analysis showed that LPS-induced Glut1 is decreased

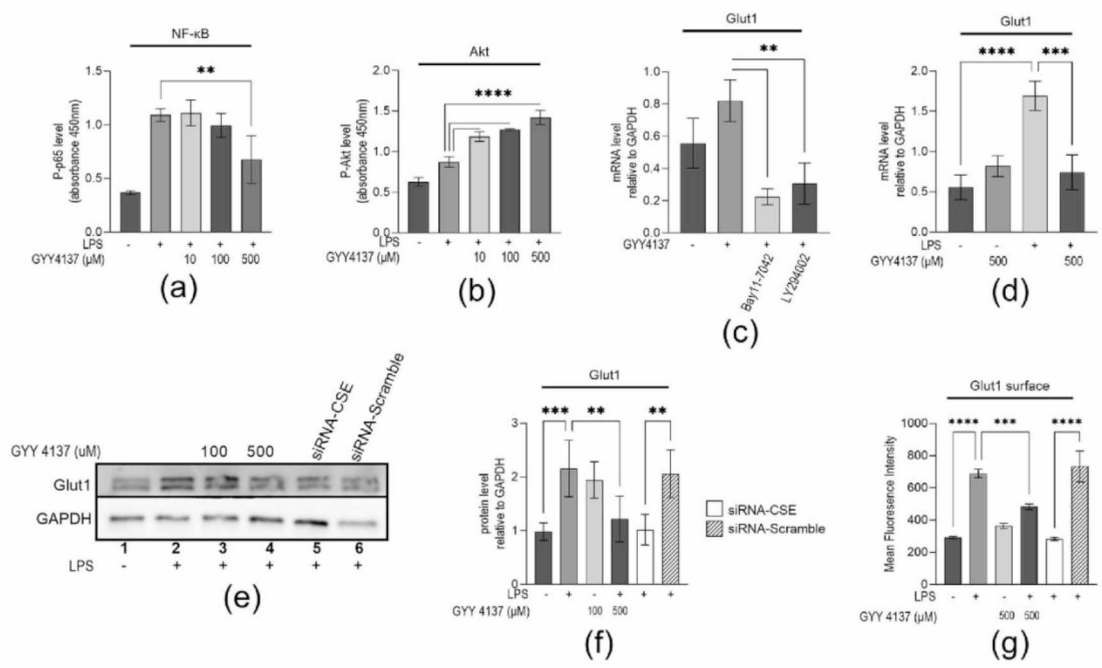

Fig 4. H_2S decreased LPS-induced NF-κB activation and increased Akt activity which is associated with Glut1 mRNA, protein, and surface expression on macrophages. (**a**–**b**) RAW264.7 cells were treated simultaneously with indicated concentrations of GYY4137 and LPS. (Panel **a** and **b**) RAW 264.7 cells were analyzed by ELISA targeting Phospho-p65 (NF-κB subunit) (panel **a**) or Phospho-Ser473 (Akt subunit) (panel **b**). (**c**) RAW264.7 cells were initially treated with IKK-inhibitor Bay11-7042 (15μM) or PI3k inhibitor LY7294002 (25μM) (for 1 h) and then treated with 500μM GYY4137 for an additional 4 h for RNA. RT-qPCR analyzed RNA for the expression of Glut1 (panel **c**). GAPDH was used as a control. (**d**) RAW264.7 cells were treated with LPS and 500μM GYY4137 for 4 h for RNA. RT-qPCR analyzed RNA for the expression of Glut1 (panel **d**). GAPDH was used as a control. (**e**—**f**) RAW264.7 cells were transfected (24 h) with CSE-siRNA or Scramble-siRNA, followed by treatment with GYY4137 and LPS for 4 h. RAW264.7 cells were analyzed by Western blotting using antibodies against Glut1 and GAPDH (loading control) (panel **e**). The changes in amounts of Glut1 have been quantified by ImageJ software and shown in panel (**f**). (**g**) RAW264.7 cells were transfected (24 h) with CSE-siRNA or Scramble-siRNA, followed by treatment with GYY4137 and LPS for 4 h. RAW264.7 cells surface staining were analyzed by flow cytometry using antibodies against Glut1 and secondary antibodies conjugated with R-PE (565/590 nm). Data represent mean ± SD (n = 3); $^{**}p < 0.001$, $^{***}p < 0.0001$, $^{****}p < 0.00001$.

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upon simultaneous treatment of LPS with 500μM GYY4137 (0.74±0.15 vs. 1.70±0.18, p = 0.002) (Fig 4d). LPS increased Glut1 protein level, on Western is decreased with treatment of 500μM GYY4137 (1.22±0.43 vs. 2.17±0.53; p<0.0082) (compare lanes 2 and 4) (Fig 4e, quantification in Fig 4f). Interestingly, Glut1 protein level with the application of siRNA targeting CSE group is similarly decreased compared to scramble siRNA (1.02± 0.28 vs. 2.06± 0.44; p<0.001) (compare lanes 5, and 6) (Fig 4e, quantification in Fig 4f).

To understand the function of H_2S in Glut1, the surface expression of Glut1 on macrophages was measured by flow cytometry. We silenced CSE in RAW264.7 cells by siRNA and scramble-siRNA and then treated with LPS without or with 500μM GYY4137. The cells were then stained with Glut1 antibody followed by R-phycoerythrin-conjugated secondary antibody. Cells were then analyzed by flow (MUSE cell analyzer). We observed that Glut1 expression levels were low in the control cells (mean fluorescence intensity MFI = 291±13.6) and significantly increased upon LPS stimulation (689±26.3; p<0.001) (Fig 4g). Interestingly, upon CSE-knockdown (CSE-siRNA and LPS treatments), the level of LPS-induced Glut1 expression was decreased relative to scramble siRNA and LPS (282.7±10.1 vs. 732.6±96.1). The surface

level of LPS-induced Glut1 was similarly decreased following treatment with GYY4173 (GYY4137 and LPS treatments). Scramble siRNA has no significant impact on LPS-induced expression of Glut1. These results further support our observation that endogenous H_2S is required for LPS-induced Glut1 in macrophages and that high levels of H_2S negatively impact this expression.

Discussion

Inflammation is a biological response of the immune system triggered by various factors, including microbial invaders and injury [35], that induces the coordinated activation of signaling pathways regulating inflammatory mediators and immune cells [36]. This response is metabolically expensive and is fueled primarily by glucose metabolism [37]. Glut1 is the primary rate-limiting glucose transporter in macrophages and a critical regulator of macrophage inflammatory response [18, 30]. Studies in the human THP-1 macrophage cell line revealed that H_2S induced the expression of Glut1 and decreased its pro-inflammatory effect [16], suggesting the potential role of H_2S and H_2S-producing enzymes in inflammatory glucose metabolism in macrophages. Thus, the CSE/H_2S system's role in Glut1 metabolism may be a relevant target for controlling macrophage inflammation.

Based on the evidence of crosstalk between H_2S and macrophage metabolism [16], we posited that the CSE/H_2S system is involved in inflammation-induced metabolism in macrophages. We confirmed the expression of CSE and demonstrated that the inflammatory Glut1 expression is induced in macrophages upon LPS stimulation, in accordance with previous studies [16]. CSE expression in macrophages is well known to play a significant role in H_2S generation during the immune response [7–9]. However, the role of CBS for the generation of H_2S in macrophages is reported to be negligible during an inflammatory response and therefore was not investigated here [38–40]. CSE expression in macrophages highlights these systems' importance in macrophage biological activities.

NF-κB plays a central role in inflammatory signaling by regulating the transcription of many pro-inflammatory cytokines, chemokines, and inflammatory mediators [41]. We demonstrated that the inhibition of IKK by Bay11-7042 significantly reduces LPS-induced Glut1 expression, implicating NF-κB in inflammation-induced Glut1 expression. In addition, our results suggest PI3k activity is associated with LPS-induced Glut1 expression. Indeed, inhibition of PI3k activation using the PI3k inhibitor LY294002 reduced LPS-induced Glut1 expression, demonstrating its role in LPS-induced metabolic function in macrophages. The metabolic regulation of activated macrophages converges from NF-kB and Akt activities upon Hif1α, a key regulator of Glut1 expression [42]. Our Nf-kB and PI3k inhibition studies agree with previous reports indicating that PI3k/Akt and NF-κB activation control macrophage inflammatory metabolism and Glut1 expression [15, 17, 18]. Next, we investigated the role of endogenous and exogenous H_2S in influencing these pathways following macrophage LPS stimulation.

We demonstrate that knockout of the CSE gene with siRNA decreased NF-κB activation in response to LPS stimulation, which is consistent with a previous report [8]. Glut1 expression is also decreased in LPS-treated macrophages genetically silenced of CSE, suggesting an impairment of the inflammation-induced metabolic program. These observations suggest a role of CSE in LPS-induced Glut1 expression via NF-κB. The interaction between H_2S and NF-κB is poorly understood. However, modifications via S-sulfhydration of cysteine residues have been suggested elsewhere. Previously, Sen *et al.* (2012) reported that H_2S modified cysteine residues of the P65 subunit of NF-κB, which enhanced binding to anti-apoptotic genes [43]. Our CSE gene knockout studies demonstrate that CSE plays a role in NF-κB activation

and that silencing CSE suppresses NF-κB-DNA binding potential. These results show the importance of the CSE/H_2S system to support NF-κB activation and the pro-inflammatory function in macrophages. However, additional work is required to elucidate the exact mechanisms that dictate the interactions between endogenous H_2S produced by CSE and pro-inflammatory NF-κB activity.

Glucose metabolism and signaling involve many upstream regulators and signaling such as PI3k/Akt. In this study, we show that H_2S from the donor molecule, GYY4137, increases the level of activated Akt in LPS-stimulated cells. Previous studies on THP-1 macrophages have found that Akt activation stabilizes HIf1α to induce Glut1 expression [44]. In lymphoid cell line FL5.12, PI3k activity was reported to regulate Glut1 trafficking and activated Akt was sufficient to maintain glucose uptake and surface Glut1 in the absence of cytokine stimulation [33]. Akt activity was also reported to be critical for the resolution of inflammation and induce anti-inflammatory alternatively activated (M2) macrophages [45]. Indeed, CSE/H_2S system activity to stabilize nuclear translocation of HIf1α was shown to promote the resolution of inflammation and injury during colitis, and H_2S donor molecules further enhance this protection [46]. Our data support the role that H_2S donor molecules promote Akt activity and may serve an anti-inflammatory role. However, Akt activity was not directly associated with Glut1 expression or surface translocation within our study.

We show that the role of the CSE/H_2S system is implicated in the expression of Glut1 under LPS stimulation via NF κB; we next investigated whether the H_2S donor molecule, GYY4137, can alter these same systems and Glut1 expression. We show that NF-κB activation is decreased in macrophages upon treatment with 500μM GYY4137 under LPS stimulation. 10μM and 100μM GYY4137 had little effect on NF-κB activation and Glut1 expression. In addition, we show that treatment with 500μM GYY4137 decreased LPS-induced Glut1 expression. Our results show that high levels of H_2S decrease NF-κB activation, potentially attenuating the macrophage pro-inflammatory response. It was reported that under prolonged exposure (24h) to H_2S, HIF1α is stabilized, increasing Glut1 expression and decreasing inflammatory activity [16]. Our results support the conclusion that H_2S donors may serve an anti-inflammatory function due to the attenuation of NF- κB activation.

Though hydrogen sulfide has been considered as a tissue protectant in some pathological conditions, but the mechanisms of tissue protection is a point of controversy. This role of hydrogen sulfide may involve the direct actions of this molecules from the indirect downstream effects. The controversy roles of hydrogen sulfide are particularly important in inflammation research. Several groups have previously reported the anti-inflammatory activity exhibited by H_2S donor molecules upon NF-κB activity in macrophages [16, 23]. Overall, we provide evidence that CSE/H_2S system regulates inflammation and serves a role in LPS-induced Glut1 expression. Future studies will be required to elucidate how the flux through various glycolytic pathways, such as the Pentose Phosphate Pathway, is influenced by H_2S. Indeed, H_2S influence on ROS is suggested in numerous studies [47–52]. Here, we demonstrate that endogenous CSE/H_2S are important for GSH production and serve a role in antioxidant defenses. However, absent CSE, GSH levels were markedly decreased but were not associated with increased NF- κB activation within our study parameters The role of the CSE/H_2S system upon Glut1 expression suggests the potential role played by endogenous H_2S to support pro-inflammatory flux through ROS-producing glycolytic pathways [18].

Based on our H_2S-donor experiments and previous studies, we hypothesize that exogenous H_2S may exert an inhibitory influence on ROS production, inhibiting pro-inflammatory response in macrophages. Additionally, we showed that H_2S donor treatment increased Akt activation; thus, it is suggested that H_2S donors exert an anti-inflammatory role. It is necessary to investigate the role of these systems in primary macrophages, as this study is limited to the

investigation of linear mouse RAW264.7 cell line. Overall, our observations demonstrate the novel regulatory pathway that H_2S influences in macrophage immune response.

Conclusions

Our results show the critical roles of CSE and H_2S in modulating glucose metabolism in macrophages. The endogenous CSE/H_2S system supports NF-κB activation and Glut1 expression under LPS-induced inflammation in macrophages. However, contradictorily, H_2S-donor molecules are demonstrated to inhibit NF-κB and Glut1 in LPS-treated macrophages at high levels. Thus, we show a regulatory network by which H_2S may influence glucose metabolism and induce metabolic reprogramming in macrophages during inflammation. Inflammation-induced Glut1 expression requires intact NF-κB and PI3k pathways. We show that H_2S donors enhance Akt activation, a downstream target of PI3k. Both PI3k and NF-κB were demonstrated to be critical for LPS-induced Glut1 expression. Overall, H_2S and CSE display regulatory roles that may influence the inflammatory potential of macrophages.

Supporting information

S1 Raw image.
(PDF)

S2 Raw image.
(PDF)

S1 Materials.
(DOCX)

Author Contributions

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Investigation: Alex Cornwell, Samantha Fedotova, Sara Cowan, Alireza Badiei.

Supervision: Alireza Badiei.

Writing – original draft: Alex Cornwell.

Writing – review & editing: Alex Cornwell, Alireza Badiei.

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