Endogenous H$_2$S targets mitochondria to promote continual phagocytosis of erythrocytes by microglia after intracerebral hemorrhage

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**ABSTRACT**

Hematoma clearance, which is achieved largely by phagocytosis of erythrocytes in the hemorrhagic brain, limits injury and facilitates recovery following intracerebral hemorrhage (ICH). Efficient phagocytosis critically depends on the capacity of a single phagocyte to phagocytize dead cells continually. However, the mechanism underlying continual phagocytosis following ICH remains unclear. We aimed to investigate the mechanism in this study. By using ICH models, we found that the gasotransmitter hydrogen sulfide (H$_2$S) is an endogenous modulator of continual phagocytosis following ICH. The expression of the H$_2$S synthase cystathionine $\beta$-synthase (CBS) and CBS-derived H$_2$S were elevated in brain-resident phagocytic microglia following ICH, which consequently promoted continual phagocytosis of erythrocytes by microglia. Microglia-specific deletion of CBS delayed spontaneous hematoma clearance via an H$_2$S-mediated mechanism following ICH. Mechanistically, oxidation of CBS-derived endogenous H$_2$S by sulfide-quinone oxidoreductase initiated reverse electron transfer at mitochondrial complex I, leading to superoxide production. Complex I-derived superoxide, in turn, activated uncoupling protein 2 (UCP2) to promote microglial phagocytosis of erythrocytes. Functionally, complex I and UCP2 were required for spontaneous hematoma clearance following ICH. Moreover, hyperhomocysteinemia, an established risk factor for stroke, impaired ICH-enhanced CBS expression and delayed hematoma resolution, while supplementing exogenous H$_2$S accelerated hematoma clearance in mice with hyperhomocysteinemia. The results suggest that the microglial CBS-H$_2$S-complex I axis is critical to continual phagocytosis following ICH and can be targeted to treat ICH.

1. Introduction

Phagocytic clearance of apoptotic cells prevents inflammation and promotes repair after tissue injury [1,2]. For efficient phagocytosis, a single phagocyte must continually take up multiple apoptotic cells [3–5]. While there has been great progress in understanding the mechanisms underlying the recognition of apoptotic cells by phagocytes, how continual phagocytosis is regulated in pathological settings is poorly understood.

Intracerebral hemorrhage (ICH) is a devastating disease with the highest mortality among all stroke subtypes and lacks effective therapies [6]. Initiated by the rupture of cerebral vessels, ICH is characterized by
the entry of blood components into the cerebral parenchyma. The deposited blood (hematoma) in the brain results in deformation of local tissue. Erythrocytes are the major component of hematoma, and erythrocyte lysis further elicits inflammatory injury and exacerbates outcomes from ICH [7]. Thus, rapid and efficient clearance of erythrocytes limits brain injury and facilitates functional recovery following ICH [8–10]. Spontaneous hematoma absorption occurs in both ICH patients and animal models [9,11–13]. In the hemorrhagic environment, erythrocytes externalize phosphatidylserine (PtdSer), a well-established marker of cell death, and facilitation post-IHC functional recovery [11]. Representative photomicrographs showing that CBS expression (red) was enhanced throughout the hematoma and perihematoma areas and that activated phagocytes (microglia/macrophages: Iba1+ cells) were present only in the perihematoma area at 3 days after cICH. (D) Representative fluorescence images showing the enhanced expression of CBS and the colocalization of CBS (red) and Iba1 (green) signals in the perihematoma area at 3 days following cICH (3 independent experiments). (E) Western blot analysis of CBS protein levels in primary microglia co-treated with lysates of red blood cells (lysate) and intact red blood cells (RBC) for 2–8 h (n = 3). Ctrl: control microglia treated with vehicle for RBC lysis. (F) Western blot analysis of CBS protein levels at 6 h after treatment with RBC lysate in primary microglia derived from the brains of wild-type mice (WT), control mice (Chsfl/fl or Cx3cr1CreER, Chsfl/fl mice (representative image from 3 independent replicates). (G and H) Representative fluorescence images and quantitative analysis of endogenous H2S production in primary microglia treated with RBCs and/or RBC lysis for 6 h using a H2S fluorescence probe (n = 9 from 3 independent experiments).

Fig. 1. CBS-derived H2S production is enhanced in microglia following ICH.

(A) CBS protein levels analyzed by Western blot in the hemorrhagic striatum following collagenase-induced ICH (cICH, n = 3). (B) H2S synthesis activity in homogenates harvested from the ipsilateral (hemorrhagic, ipsi) and contralateral (Contra) striatum following cICH (n = 3 or 4). Sham: sham-operated mice. (C) Representative primary microglia showing that CBS expression (red) was enhanced throughout the hematoma and perihematoma areas and that activated phagocytes (microglia/macrophages: Iba1+ cells) were present only in the perihematoma area at 3 days after cICH. (D) Representative fluorescence images showing the enhanced expression of CBS and the colocalization of CBS (red) and Iba1 (green) signals in the perihematoma area at 3 days following cICH (3 independent experiments). (E) Western blot analysis of CBS protein levels in primary microglia co-treated with lysates of red blood cells (lysate) and intact red blood cells (RBC) for 2–8 h (n = 3). Ctrl: control microglia treated with vehicle for RBC lysis. (F) Western blot analysis of CBS protein levels at 6 h after treatment with RBC lysate in primary microglia derived from the brains of wild-type mice (WT), control mice (Chsfl/fl or Cx3cr1CreER, Chsfl/fl mice (representative image from 3 independent replicates). (G and H) Representative fluorescence images and quantitative analysis of endogenous H2S production in primary microglia treated with RBCs and/or RBC lysis for 6 h using a H2S fluorescence probe (n = 9 from 3 independent experiments).

2. Materials and methods

2.1. Intracerebral hemorrhage models and drug administration

The work was performed under project licenses of grants from the National Natural Science Foundation of China (81971119, 82071469, 81571124 and 81671310). The protocols were approved by the Animal Care and Use Committee of Soochow University. Chsfl/fl and Sqrfl/fl mice were generated by Shanghai Model Organisms and were transferred, maintained and backcrossed for more than 9 generations to a C57BL/6 background at Soochow University. Chsfl/fl and Sqrfl/fl mice were crossed with Cx3cr1Cre or Cx3cr1CreER mice (Jackson Laboratory). Cx3cr1CreER; Chsfl/fl mice were intraperitoneally injected with TAM (100 mg/kg/day) for 5 days to generate mice with microglia-specific knockout of CBS, as reported [19]. ROSA-tdtomato mice (Jackson Laboratory) were crossed with Cx3cr1CreERT2 mice, followed by TAM injection. Gene engineered mice were bred under specific pathogen-free (SPF) conditions. Wild-type ICR mice were purchased from SLAC Laboratory (Shanghai, China). Male adult mice (2 months old), weighing 25–30 g, were used in the study. All mice were kept with a 12 h circadian cycle in a temperature-controlled environment and had ad libitum access to water and food. ICH was induced in mice by injecting collagenase VII-S (0.03 Units; Sigma–Aldrich) or autologous blood (15 μL) was placed into the left striatum under anesthesia using a microinfusion pump (RWD Instruments, Shenzhen, China), as we described [20,21]. The coordinates relative to bregma were 0.5 mm anterior to bregma, 2 mm lateral to the midline and 3.5 mm below the skull. Sham-operated mice received the same volumes of saline. Mice were randomized to receive a daily intraperitoneal injection of the H2S donor ADT (20 mg/kg/day) or 5a (0.5 mg/kg/day) starting at 3 h after ICH. The sample sizes were estimated based on our previous experience. No animal was excluded from the analysis.

2.2. Hemorrhagic volume assessment and behavioral tests

At the timepoints indicated in the figure legends, ICH mice were transcranially perfused with phosphate buffered saline (PBS). Drabkin reagent (80 μL, Sigma–Aldrich) was added to a 20-μL aliquot of the hemorrhagic brain tissue homogenates. Optical density was assayed at a wavelength of 540 nm. Hemorrhagic volumes were expressed as the volume of blood (μL)/hemisphere by comparison with a standard curve made from fresh blood as reported [12].

For general neurologic severity assessment, each mouse was given points based on the following tests: gait, whisker response, body symmetry, symmetry in front limbs, climbing, circling behavior, and compulsive circling [21]. The maximum score of 24 indicated the most severe deficit. The corner turn test was performed by forcing mice into a corner. Mice preferentially turned toward the nonimpaired side following ICH. The results are expressed as the percentages of left turns to total trials. Normal mice can place the forelimb ipsilateral to vibrissae stimulation on the countertop. ICH impairs the placement of the forelimb contralateral to the hemorrhagic brain [20]. The forelimb placement test was repeated 10 times/mouse [20]. The number of trials in which mice appropriately placed their forelimbs was recorded and expressed as the percentage of total trials [20].

2.3. Primary microglia and lentiviral infection

Cortical tissue was harvested from 1-day-old newborn mice (C57BL/6J). The cortices were kept in icy DMEM/F12 medium and digested with EDTA-trypsin for 10 min. Digestion was terminated with DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS). The cell suspension was filtered through a 50 μm cell strainer to remove tissue debris. Cells were collected and plated in flasks precoated with poly-l-lysine. Cells were cultured with medium replacement every 3 days. After culturing in vitro for 10–12 days, flasks were shaken at 180 rpm for 30 min. Floating microglia were harvested and seeded on 24-well plates.
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After microglial cultures reached 90% confluence, cells were infected with lentivirus coexpressing green fluorescence protein (GFP) and shRNA against NADH: ubiquinone oxidoreductase core subunit S3 (NDUFS3) or UCP2 (GeneChem Shanghai, China) at a multiplicity of infection of 4. The NDUFS3-shRNA and UCP2-shRNA sequences were validated in our previous publication [20]. Infected cells were used for further experiments or for the analysis of knockdown efficiency at 4 days after infection.

2.4. In vitro erythropagocytosis by microglia

Mouse primary microglia were seeded on 24- or 96-well plates overnight. Red blood cells (RBCs) were isolated from mouse whole blood via centrifugation, as reported [22]. After labeling with the fluorescent probe PKH-26 (Sigma–Aldrich), RBCs were fed to microglia at a ratio of 10:1 with/without RBC lysate for 6 h. To prepare RBC lysate, packed RBCs were frozen in liquid nitrogen and then allowed to thaw at 37 °C for 3 cycles, as described [20,23]. Unphagocytosed RBCs in culture medium were counted. Microglia containing phagocytosed RBCs were lysed with distilled water. Fluorescence intensity was assayed with a 492/527 nm filter set and expressed as the phagocytosis index, i.e., the ratio to fluorescence intensity inside control microglia that were not lysed with distilled water. Fluorescence intensity was assayed with a fluorescence microscope (Zeiss LSM700, Germany).

2.5. Assessment of H2S synthesizing activity

H2S synthesis activity in the brain hemisphere was assessed as we described [24]. At the timepoints indicated in the figure legends, the brain hemisphere was homogenized in potassium phosphate buffer (50 mM/mL). Homogenates were kept in 20-ml glass vials and cooled on ice for 10 min. Then, 1-cysteine (10 mM/mL) and pyridoxal 5-phosphate (2 mM/mL) were added. Next, a 2-ml tube, which contained a piece of filter paper soaked with zinc acetate (1%; 0.3 mL), was placed inside the vial. The vial was flushed with nitrogen gas for 20 s, capped and kept in a shaking water bath. After 90 min of incubation, trichloroacetic acid (TCA, 50%; 0.5 mL) was injected through the cap into the vial. At 60 min after TCA addition, FeCl3 (30 mM/mL; 50 μL) in 1.2 mol/L HCl and N,N-dimethyl-p-phenylenediamine sulfate (20 mM/mL; 50 μL) in 7.2 mol/L HCl were added to the inner tube. After 20 min, the absorbance at 670 nm was measured using a TECAN, Mannendorf, Switzerland. H2S concentrations were calculated against a standard curve made from NaHS. The velocity of H2S synthesis was expressed as nmol H2S/g tissue/h.

2.6. Assessment of endogenous H2S levels in primary microglia

Endogenous H2S levels were indicated by the fluorescence probe HSip-1 DA [25]. Mouse primary microglia were cultured on 24-well plates in DMEM supplemented with 10% FBS at 37 °C overnight. After 3 washes with FBS-free DMEM, the cells were incubated in medium containing HSiP-1 DA (10 μM, Dojindo Molecular Technologies, Japan) at 37 °C for 30 min. Then, RBC lysate (10 μL) was added to the medium, and the cells were kept at 37 °C for 6 h. After removal of the probe and washing with PBS (pH 7.4), cell imaging was performed under a fluorescence microscope (Zeiss LSM700, Germany).

2.7. Western blot

Cellular and tissue samples were lysed in RIPA lysis buffer (NCM Biotech, Sookchow, China) supplemented with phosphatase and protease inhibitors. Total protein concentrations were measured using a commercial kit (Beyotime, Shanghai, China, P0011). Equal amounts of lysates (40 μg protein/sample) were mixed with loading buffer and boiled for 10 min. Proteins were separated by SDS-PAGE electrophoresis and transferred to polyvinylidene difluoride membranes. After blocking in buffer containing 5% nonfat milk and 0.1% Tween-20, the membranes were probed with primary antibodies against CBS (Proteintech, Wuhan, China, 14787-1-AP; RRID: AB_2070973); or Santa Cruz, Cat #: SC-67154; RRID: AB_2070973) [26], UCP2 (Santa Cruz, Dallas Texas, USA, sc-390189; RRID: AB_2721285) [27], NDUFS3 (GeneChem Shanghai, China, Cat# DP419). Protein bands were visualized with a chemiluminescent reagent (NCM Biotech, P10300A, China). Images were captured with a chemiluminescence imaging system (Bio-Rad, CA, USA) and semi-quantified with ImageJ software.

2.8. Quantitative PCR

Tissue samples were harvested from the ipsilateral and contralateral striatum at 3 days following ICH or the striatum of sham-operated mice. Total RNA was isolated from the tissue samples using a commercial kit (RNAsimple Total RNA Kit, Tiangeng, Shanghai, China, Cat# DP419). Reverse transcription was performed using 1 μg total RNA and a high-capacity cDNA synthesis kit (Applied Biosystem, Foster City, CA). Quantitative PCR (qPCR) assays were performed with a real-time SYBR technique (ChamQ Universal SYBR qPCR Master Mix, Vazyme, Nanjing, China, QT71-02) using an ABI Prism 7000 DNA Detection System. qPCR primers for the CBS mRNA assay were 5′- AGGGCTATCGCTGCAT -3′ (forward) and 5′- AGGTCTACCATGACGCTCTC -3′ (reverse). For each sample, GAPDH RNA was also assessed as an internal control.

2.9. Measurement of mitochondrial membrane potential

To assess ΔΨm, the fluorescent probes JC-1 or tetramethylrhodamine ethyl ester (TMRM, Sigma–Aldrich, St. Louis, MO, USA) was used to stain cultured cells as reported [29]. JC-1 diffuses into the plasma from the mitochondrion when ΔΨm decreases, as indicated by the shift in fluorescence emission from red to green. Microglia seeded on 24-well plates (10 [5] cells/well) were incubated in medium containing 100...
2.10. Assessment of mitochondrial ROS generation in cultured cells

Mitochondrial ROS were assessed using the fluorescent probe Mitosox (5 μM, Thermo Fisher Scientific, Carlsbad, CA, USA), as we described [20]. Microglia were incubated in medium containing Mitosox (5 μM, Thermo Fisher Scientific, Carlsbad, CA, USA) and Mitotracker Green FM (50 nM, Thermo Fisher Scientific, Carlsbad, CA, USA). After 3 washes in PBS, microglia were further incubated with Hoechst 33342 (10 μM, Sigma Aldrich) to stain nuclei. Fluorescence images were taken under confocal microscopy (Zeiss LSM700, Germany) with constant parameters. ImageJ software (http://rsbweb.nih.gov/ij/) was used for semi-quantification.

2.11. Lentiviral knockdown of target genes in the striatum

As we reported previously [20,21], lentiviruses were stereotaxically injected into the left striatum to knock down target genes. In brief, anesthetized mice were placed onto a stereotactic apparatus. Lentiviruses expressing short hairpin RNA (shRNA) against UCP2 or NDUF53 (GeneChem, Shanghai, China) were injected into the two sites of the left striatum at a rate of 0.5 μL/min with a 30-gauge needle (1.5 μL of 1 × 10^6 TU/mL lentivirus per injection site). The control mice received lentivirus expressing nontargeted shRNA (NC-shRNA). UCP2-shRNA and NDUF53-shRNA were validated in our previous publication [20,21]. The coordinates were 0.5 or 1 mm anterior to bregma, 2.0 or 1.5 mm lateral to the midline, and 3.5 or 3.2 mm below the skull. The mice were subjected to ICH surgery at 14 days after viral injection.

2.12. Tissue histochemistry and assessment of injury volumes following ICH

At the timepoints indicated in the figure legends, mice were transcardially perfused with 0.1 mol/L PBS and then perfused with 4% PFA. Brains were collected and cut into coronal sections (25 μm) on a cryostat. From the onset of hemorrhage, one of every 11 sections was subjected to ICH surgery at 14 days after viral injection. The mice were randomized into 2 groups. Control mice were fed a normal chow diet. Methionine-treated mice were fed a chow diet supplemented with 2% methionine in drinking water (w/v) for 2 weeks.

2.13. Induction of hyperhomocysteinemia in mice

Hyperhomocysteinemia was induced in mice by feeding them methionine, as we described [31]. Six-week-old male ICR mice were randomly divided into 2 groups. Control mice were fed a normal chow diet. Methionine-treated mice were fed a chow diet supplemented with 2% methionine in drinking water (w/v) for 2 weeks.

2.14. Statistical analysis

Data are expressed as the mean ± s.e.m. and were analyzed using SPSS Statistics 17.0. Two-tailed Student’s t tests were used for pairwise comparisons. Multiple comparisons were performed using one-way ANOVA followed by post hoc Tukey analysis if the results were similar. Otherwise, data were analyzed by Games–Howell’s test. Behavioral data were analyzed by two-way ANOVA. P < 0.05 was considered statistically significant.

2.15. Data availability

All raw data are available from the corresponding authors upon request.

3. Results

3.1. CBS-derived endogenous H_2S production is enhanced in microglia following ICH

CBS polymorphisms are independently associated with outcomes in patients with subarachnoid hemorrhage [16]. However, whether CBS is involved in ICH pathogenesis remains unclear. The Western blot results from two kinds of mouse ICH models, i.e., collagenase-induced ICH and autologous blood-induced ICH in the left striatum, showed an increase in CBS protein levels in the ipsilateral (hemorrhagic) striatum at 2–5 days following ICH (Fig. 1A and Supplementary Figs. 1A–B). Since the CBS antibody generated multiple protein bands, we validated the CBS protein band using brain tissue harvested from mice with CBS deficiency in microglia/macrophages (Supplementary Fig. 1C). Moreover, CBS mRNA was also enhanced in the hemorrhagic (Ipsil) striatum vs. the contralateral striatum or the striatum of sham-operated mice, suggesting that CBS expression was enhanced following ICH via a transcriptional mechanism (Supplementary Fig. 1D). Consistently, the H_2S synthesis activity in the homogenate of the ipsilateral striatum was also increased at 3 and 5 days after ICH (Fig. 1B). Immunofluorescence results further showed that CBS protein levels were elevated in the hematoma center and perihematoma area of the ipsilateral striatum vs. the contralateral striatum at 3 days following ICH (Fig. 1C and D). ICH induced activation of microglia/macrophages in the ipsilateral striatum, as indicated by the
amoeboid morphology of Iba1+ cells (Fig. 1D). Consistent with published results [12], microglia/macrophages were present only in the perihematoma area (Fig. 1C). Importantly, ICH-enhanced expression of CBS in the perihematoma area mainly colocalized with activated microglia/macrophages (Fig. 1D), suggesting that microglia/macrophages were important cellular sources for ICH-enhanced expression of CBS.

Lysis of RBCs starts from the hematoma center at 1 day and is evident at 3 days following ICH [32], which largely correlates with the time course of ICH-enhanced CBS expression. Thus, we hypothesized that the lysate of RBCs enhanced CBS expression in microglia. In line with this hypothesis, RBC lysate enhanced CBS protein expression in primary microglia (Fig. 1E). Importantly, treating microglia with intact RBCs alone did not enhance CBS expression (Supplementary Figs. 1C and D).

We further generated mice with CBS deficiency in microglia/macrophages (Cx3cr1Cre; Cbsfl/fl) and prepared primary microglia from Cx3cr1Cre; Cbsfl/fl mice. CBS expression was null in Cx3cr1Cre; Cbsfl/fl microglia (Fig. 1F). Importantly, RBC lysate elevated CBS expression in wild-type and control microglia (Cbs+/+) but not in Cx3cr1Cre; Cbsfl/fl microglia (Fig. 1F). By using a fluorescence probe specific for H2S [25], we further showed that treating microglia with RBC lysate did not enhance endogenous H2S production in CBS-deleted microglia but robustly enhanced H2S production in WT and control microglia (Fig. 1G and H). Notably, intact RBCs did not enhance endogenous H2S production in wild-type and control microglia (Fig. 1G and H). To conclude, RBC lysate enhanced endogenous H2S production in microglia in a CBS-dependent manner following ICH.

Moreover, we examined the expression of cystathionine gamma lyase (CSE) and 3-mercaptopropionate sulfurrtransferase (3-MST), which are two other important H2S synthases, following ICH. We found that the expression of CSE, but not that of 3-MST, was enhanced in the hemorrhagic striatum following ICH (Supplementary Figs. 2A–D). Immunohistochemistry further showed that neurons, but not microglia, were the major cellular source responsible for CSE expression both in the hemorrhagic and contralateral striatum (Supplementary Figs. 2E and F). This suggested that CSE might not contribute to microglial phagocytosis of erythrocytes after ICH. Collectively, the results suggested that CBS-derived H2S was enhanced in microglia following ICH.

3.2. Microglial CBS facilitates spontaneous hematoma clearance via an H2S-mediated mechanism following ICH

We further investigated whether CBS in microglia/macrophages affected spontaneous hematoma clearance following ICH by using CBS-deleted mice (Cx3cr1Cre; Cbsfl/fl). Although the initial hemorrhagic volumes at day 2 after ICH were comparable between Cx3cr1Cre; Cbsfl/fl and control Cbs+/+ mice (Supplementary Fig. 3A), Cx3cr1Cre; Cbsfl/fl mice exhibited larger residual hematomas than Cbs+/+ mice at day 5 after collagenase-induced ICH (Fig. 2A and B). By day 14 after ICH, a time point when almost all hematomas had been cleared in the control mice, residual hematomas were still visible in Cx3cr1Cre; Cbsfl/fl mice (Fig. 2C).

Moreover, hemorrhagic-induced brain injury was also exacerbated in Cx3cr1Cre; Cbsfl/fl mice (Supplementary Figs. 3B and C). To conclude, CBS deficiency in microglia/macrophages delayed spontaneous hematoma clearance following ICH.

CBS displays multiple functions [33]. To investigate whether CBS acted through H2S to contribute to hematoma clearance, we examined whether exogenous supplementation with H2S rescued CBS deletion-induced impairment in spontaneous hematoma clearance. To do this, we used the clinical drug anethole trithione (ADT) since ADT is a slow-releasing H2S donor, and H2S release from ADT and its in vivo metabolite ADT-OH requires intracellular enzymes, which mimic the endogenous production of H2S [34]. Moreover, ADT and AT-OH better mimic the actions of endogenously produced H2S than the inorganic donor NaHS [20,24,35]. Compared to vehicle injection, ADT injection, starting at 3 h after ICH, reduced hematoma volumes (Supplementary Figs. 3D and E) and improved neurological deficits in Cx3cr1Cre; Cbs+/+ mice (Supplementary Figs. 3F–H).

To discriminate the role of CBS in brain-resident phagocytic microglia versus peripheral phagocytes, we used Cx3cr1CreER mice, which express Cre recombinase via a tamoxifen-inducible mechanism and have been used to specifically delete target genes in microglia [19]. We generated Cx3cr1CreER; Cbsfl/fl mice (Fig. 2D). Compared to control Cx3cr1CreER; Cbs+/+ mice that were injected with vehicle (oil), mice with microglia-specific deletion of CBS (Cx3cr1CreER; Cbsfl/fl mice receiving tamoxifen) displayed larger hemorrhagic volumes following ICH (Fig. 2E–G). Moreover, ADT rescued microglial CBS deletion-induced impairment in hematoma resolution (Fig. 2H and I) and improved neurological deficits (Fig. 2, J–L) in Cx3cr1CreER; Cbsfl/fl mice receiving tamoxifen. Together, the results suggested that microglial CBS facilitated spontaneous hematoma resolution via an H2S-mediated mechanism. In contrast, CBS in peripheral phagocytes contributed little to hematoma clearance.

3.3. Microglial CBS promotes continual erythrophaocytosis in an H2S-dependent manner

Microglia must be activated to phagocitize erythrocytes following ICH [14]. To model erythrophaocytosis by microglia under hemorrhagic conditions, we incubated primary microglia with fluorescently labeled RBCs and simultaneously cotreated microglia with RBC lysate to mimic ICH-induced microglial activation [20,35] as well as ICH-enhanced expression of CBS. Compared to vehicle, RBC lysate promoted erythrophaocytosis by microglia at 6 h after microglia were exposed to RBCs (Fig. 3A–C). Microglial phagocytosis of RBCs via a PtdSer-dependent mechanism under hemorrhagic conditions both in vitro and in vivo [14]. Consistently, annexin V, an inhibitor that blocks the interaction between dead cell-externalized PtdSer and its receptors, dose-dependently inhibited the phagocytosis of RBCs by microglia treated with RBC lysate (Supplementary Figs. 4A and B). ZnCl2 blocks the effects of free H2S [20]. Notably, ZnCl2 attenuated the promoting effects of RBC lysate on microglial erythrophaocytosis (Fig. 3A–C). We further isolated primary microglia from CBS-deleted mice (Cx3cr1CreER; Cbsfl/fl) and control mice (Cbs+/+). Compared to control microglia, CBS-deleted microglia showed less phagocytosis of RBCs following treatment with RBC lysate (Supplementary Fig. 4C). Moreover, ADT rescued CBS deletion-induced impairment in erythrocyte effrophagocytosis by Cx3cr1CreER; Cbsfl/fl microglia (Fig. 3D and E).

Due to the limited survival of phagocytes vs. neutrophils under pathological conditions, continual phagocytosis is critical to efficient phagocytosis [3,4]. We constructed Cx3cr1CreER; Rosa26tdTomato mice to specifically label microglia with tomato fluorescence, as previously reported [19]. As expected, a relatively small number of microglia (Tomato+ cells) surrounded the large volumes of hematoma at 3 days...
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following ICH (Fig. 3F). Thus, we investigated whether ICH-enhanced expression of CBS enabled microglia to internalize RBCs continually by using a two-stage approach [3]. Briefly, the first round and second round of RBCs to be phagocytized were prelabeled with PKH26 (red fluorescence) and PKH67 (green fluorescence), respectively. The percentage of CBS-deleted microglia that internalized red fluorescence was not different from the percentage of control microglia that internalized red fluorescence (Fig. 3G and H). In contrast, the percentage of CBS-deleted microglia that internalized both types of fluorescence was remarkably lower than the percentage of control microglia that internalized both types of fluorescence (Fig. 3G and H). The results indicated that CBS deficiency impaired continual phagocytosis rather than the first round of phagocytosis. Thus, CBS-derived endogenous H\textsubscript{2}S promoted continual phagocytosis of RBCs by microglia.

3.4. CBS-derived H\textsubscript{2}S acts via SQR to promote microglial phagocytosis and hematoma resolution after ICH

Continual phagocytosis is remarkably enhanced by the reduction in mitochondrial membrane potential (ΔΨ\textsubscript{m}) [4]. RBC lystate, but not intact RBCs, reduced ΔΨ\textsubscript{m} in the WT microglia, and the reduction was blocked by ZnCl\textsubscript{2} (Supplementary Figs. 5A and B). Moreover, RBC lystate reduced ΔΨ\textsubscript{m} in the control microglia but not in CBS-deleted microglia (Supplementary Figs. 5C and D). Together, the results suggested that CBS-derived endogenous H\textsubscript{2}S reduced ΔΨ\textsubscript{m} in microglia treated with RBC lystate.

The expression of UCP2, a protein that dissipates ΔΨ\textsubscript{m} is upregulated in phagocytes engulfing apoptotic cells, and upregulated UCP2 consequently reduces ΔΨ\textsubscript{m} to promote continual phagocytosis [4]. However, UCP2 protein levels were not enhanced in microglia that were exposed to RBCs and simultaneously treated with RBC lystate (Supplementary Figs. 5E and F). Notably, UCP2 displays a weak capacity to dissipate ΔΨ\textsubscript{m} unless it is activated by mitochondrial ROS [36,37]. We reported that oxidation of exogenous H\textsubscript{2}S by SQR enhanced mitochondrial production of ROS by initiating reverse electron transfer at mitochondrial complex I (complex I RET) [20]. Thus, CBS-derived endogenous H\textsubscript{2}S might be oxidized by SQR to generate mitochondrial ROS, and ROS in turn activate UCP2 to promote continual erythrophagocytosis by microglia. To test this hypothesis, we first examined whether SQR was required for endogenous H\textsubscript{2}S to enhance microglial erythrophagocytosis. We generated mice with deletion of SQR in microglia/macrophages (Cx3cr1\textsuperscript{Cre}: Sqr\textsuperscript{fl/fl}) as we reported previously [20] and prepared primary microglia from Cx3cr1\textsuperscript{Cre}: Sqr\textsuperscript{fl/fl} mice. Deletion of SQR abolished the RBC lystate-induced reduction in ΔΨ\textsubscript{m} in microglia regardless of the presence or absence of RBCs (Fig. 4A and B). Compared to control microglia (Sqr\textsuperscript{Wt}/Wt), Cx3cr1\textsuperscript{Cre}: Sqr\textsuperscript{fl/fl} microglia showed less phagocytosis of RBCs following treatment with RBC lystate (Fig. 4C and D). Notably, ADT did not rescue SQR deletion-induced impairment in erythrophagocytosis by Cx3cr1\textsuperscript{Cre}: Sqr\textsuperscript{fl/fl} microglia following treatment with RBC lystate (Fig. 4C and D). Cx3cr1\textsuperscript{Cre}:Sqr\textsuperscript{fl/fl} mice also displayed larger hemorrhagic volumes (Fig. 4E and F) and more severe neurological deficits (Fig. 4G–I) than control mice following ICH. Moreover, the H\textsubscript{2}S donor ADT neither rescued SQR deletion-induced impairment in spontaneous hematoma resolution nor facilitated behavioral recovery in Cx3cr1\textsuperscript{Cre}: Sqr\textsuperscript{fl/fl} mice (Fig. 4E–I). ADT itself had no effect on the striatal expression of SQR in the absence of ICH (Fig. 4J). To conclude, SQR was required for CBS-derived endogenous H\textsubscript{2}S to promote microglial erythrophagocytosis and spontaneous hematoma clearance following ICH.

3.5. Oxidation of CBS-derived H\textsubscript{2}S by SQR promotes spontaneous hematoma clearance via complex I-derived ROS

Next, we tested the hypothesis that oxidation of CBS-derived endogenous H\textsubscript{2}S by SQR promoted hematoma clearance via complex I-derived ROS. RBC lystate enhanced mitochondrial ROS levels in the WT and control microglia (Cbs\textsuperscript{fl/fl}) but not in CBS-deleted microglia, regardless of the presence or absence of intact RBCs (Fig. 5A). Moreover, RBC lystate-enhanced production of mitochondrial ROS was blocked by ZnCl\textsubscript{2} (Supplementary Fig. 6A). Together, the results suggested that CBS-derived endogenous H\textsubscript{2}S enhanced mitochondrial ROS production in microglia treated with RBC lystate. Moreover, MitoTempo, a mitochondrial-targeted ROS scavenger [20], blocked the promoting effects of RBC lystate on mitochondrial phagocytosis of RBCs (Supplementary Figs. 6B and C), further suggesting that mitochondrial ROS were required for endogenous H\textsubscript{2}S to promote microglial phagocytosis of RBCs.

We reported that oxidation of exogenous H\textsubscript{2}S by SQR repurposed mitochondrial function to ROS production by initiating complex I RET [20]. The hallmark property of SQR-induced complex I RET is that the H\textsubscript{2}S-induced mitochondrial ROS burst is blocked by the inhibition of complex I [20]. RBC lystate enhanced mitochondrial ROS levels in WT and control microglia (Sqr\textsuperscript{fl/fl}) but not in SQR-deleted microglia (Fig. 5B). Moreover, the burst of mitochondrial ROS in microglia treated with RBC lystate was attenuated by the complex I inhibitor rotenone (Supplementary Fig. 6D). In addition, we knocked down the mitochondrial complex I subunit NDUF53 in microglia by using a lentivirus expressing NDUF53-shRNA [20]. Knockdown of NDUF53 also attenuated the mitochondrial ROS burst induced by RBC lystate in microglia (Fig. 5C). Together, the results suggested that enhanced production of endogenous H\textsubscript{2}S in microglia treated with RBC lystate initiated complex I RET via SQR, which consequently repurposed mitochondrial function to ROS production. Notably, high ΔΨ\textsubscript{m} is required for the initiation of complex I RET [38]. Although 6 h of treatment with RBC lystate decreased ΔΨ\textsubscript{m} in microglia via CBS-derived endogenous H\textsubscript{2}S, 4.5 h of treatment with RBC lystate enhanced ΔΨ\textsubscript{m} (Supplementary Figs. 6E and F). Consistently, exogenous H\textsubscript{2}S also elevates ΔΨ\textsubscript{m} to initiate complex I RET before it decreases ΔΨ\textsubscript{m}. Together, the results suggested that oxidation of endogenous H\textsubscript{2}S by SQR elevated mitochondrial ROS by initiating complex I RET.

Next, we asked whether complex I functionally contributed to spontaneous hematoma clearance following ICH. Rotenone abolished the reduction in ΔΨ\textsubscript{m} induced by RBC lystate (Supplementary Fig. 6G) and blocked the promoting effects of RBC lystate on mitochondrial phagocytosis of RBCs (Supplementary Figs. 6H and I). Knockdown of NDUF53 also abolished the RBC lystate-induced reduction in ΔΨ\textsubscript{m} (Fig. 5D and E).
Fig. 6. Activation of UCP2 by ROS is required for endogenous H$_2$S to facilitate microglial phagocytosis of RBCs and spontaneous hematoma resolution following ICH. (A and B) Representative fluorescence images and quantification data showing that the RBC lysate-induced reduction in $\Delta$Ψ$_m$ in primary microglia was blocked by genipin, an inhibitor of ROS-mediated activation of UCP2 (JC-1 staining, $n=9$ from 3 independent biological replicates). (C and D) Genipin inhibited the engulfment of RBCs by primary microglia treated with RBC lysate, as indicated by the number of nonphagocytosed RBCs and the phagocytosis index ($n=3$). (E and F) Quantification data and representative fluorescence images showing that knockdown of UCP2 in microglia blocked the reduction in $\Delta$Ψ$_m$ induced by RBC lysate in primary microglia (TMRE staining, $n=9$ from 3 independent replicates). (G) Immunoblot analysis of UCP2 expression in the striatum transfected with a lentivirus expressing UCP2-shRNA or NC-shRNA for 14 days (representative images from 3 independent experiments). Ipsil: the striatum ipsilateral to lentiviral injection. Contra: the contralateral striatum. (H and I) Striatal knockdown of UCP2 delayed spontaneous hematoma resolution following ICH, and the delay was not rescued by the H$_2$S donor ADT, as indicated by hemorrhagic volumes at days 5 and 14 following ICH ($n=6$).

and blunted the promoting effects of RBC lysate on microglial phagocytosis of RBCs (Supplementary Figs. 6J and K). Moreover, we injected lentiviruses expressing NDUFS3-shRNA into the left striatum to knock down NDUFS3 in vivo (Fig. 5F). Compared to the control lentivirus, the lentiviruses expressing NDUFS3-shRNA increased residual hematoma volumes (Fig. 5G and H) and delayed functional recovery following ICH (Fig. 5I–K). Notably, ADT did not rescue the impairment in spontaneous hematoma resolution or the delay in functional recovery induced by striatal knockdown of NDUFS3 (Fig. 5G–K).

Finally, we investigated whether complex I-derived ROS reduced $\Delta$Ψ$_m$ and promoted microglial phagocytosis of RBCs by activating UCP2. Genipin is not an ROS scavenger but specifically inhibits ROS-mediated activation of UCP2 [39]. The RBC lysate-induced reduction in $\Delta$Ψ$_m$ in microglia was blocked by genipin (Fig. 6A and B). Moreover, genipin blocked the promoting effects of RBC lysate on microglial phagocytosis of RBCs (Fig. 6C and D). Consistently, lentiviral knockdown of UCP2 in microglia also blocked the RBC lysate-induced reduction in $\Delta$Ψ$_m$ (Fig. 6E and F) and blunted the promoting effects of RBC lysate on microglial phagocytosis of RBCs (Supplementary Figs. 7A and B). We further knocked down UCP2 in the striatum via lentivirus-expressed shRNA (Fig. 6G). Striatal knockdown of UCP2 delayed spontaneous hematoma resolution (Fig. 6H and I) and functional recovery (Supplementary Figs. 7C–E) following ICH, and the delay was not rescued by the H$_2$S donor ADT (Fig. 6H and I and Supplementary Figs. 6C–E). Moreover, ADT itself had no effect on the striatal expression of UCP2 in the absence of ICH (Supplementary Figs. 7F and G).

3.6. The CBS-H$_2$S axis can be targeted to accelerate hematoma clearance

How hyperhomocysteinemia, the established risk factor for stroke, exacerbates outcomes from ICH remains unclear. Since direct injection of homocysteine into the brain decreases CBS expression [40], we hypothesized that hyperhomocysteinemia exacerbated outcomes by inhibiting CBS expression following ICH. Hyperhomocysteinemia was induced in mice via dietary methionine supplementation, as we described [31]. CBS-enhanced expression of CBS was robustly inhibited in the mice fed methionine compared with control mice fed normal water (Fig. 7A and B). Mice fed methionine displayed larger residual hematoma volumes and more severe neurological deficits than control mice after ICH (Fig. 7C–G). The results recapitulated the impairment in hematoma resolution in CBS-deleted mice. Moreover, ADT accelerated hematoma clearance (Fig. 7C and D) and improved neurological deficits in mice fed methionine (Fig. 7E–G). The structurally unrelated H$_2$S donor 5a releases H$_2$S in the presence of intracellular cysteine [41], 5a also accelerated hematoma clearance (Supplementary Fig. 8A) and improved neurological deficits in mice fed methionine after ICH (Supplementary Figs. 8B–D). Moreover, 5a did not affect CBS expression in the hemorrhagic brain (Supplementary Figs. 8E and F), suggesting that H$_2$S supplementation had no effect on CBS expression. Collectively, the results suggested that hyperhomocysteinemia impaired CBS-enhanced CBS expression to delay hematoma resolution, while supplementing exogenous H$_2$S accelerated hematoma clearance following ICH associated with hyperhomocysteinemia.

4. Discussion

The major finding of the study is that CBS-derived H$_2$S may represent a presently unrecognized modulator of continual phagocytosis following ICH. CBS-derived H$_2$S was elevated in brain-resident phagocytic microglia after ICH. This represented a novel mechanism underlying spontaneous hematoma clearance following ICH. Nevertheless, how CBS expression is upregulated following ICH warrants further investigation. While the association of CBS with numerous diseases has been documented, little is known about the role of the H$_2$S synthesis activity of CBS in diseases [42]. Moreover, although H$_2$S is considered an important endogenous gasotransmitter, the mechanisms underlying the pathophysiological functions of endogenous H$_2$S largely remain unknown. The results from the study suggested that endogenous H$_2$S may function as a phagocytosis modulator to regulate pathophysiological processes.

CBS also functions as a key enzyme in the transsulfuration pathway that leads to the biosynthesis of glutathione (GSH). Currently, the role of GSH in hematoma resolution after ICH remains unclear. Here, we found that the promoting effects of CBS-derived H$_2$S on microglial phagocytosis of erythrocytes under hemorrhagic conditions were dependent on mitochondrial ROS (Fig. 5). Since GSH is a well-recognized scavenger of free radicals, it is unlikely that GSH plays a role in CBS-promoted hematoma resolution following ICH. Moreover, heterozygous deletion of CBS does not affect GSH levels [43]. Collectively, we provide evidence that CBS contributes to hematoma resolution by generating endogenous H$_2$S following ICH.

H$_2$S is highly diffusible. However, our results suggested that endogenous H$_2$S produced in microglia might only serve as an intracellular signaling molecule. CBS expression was enhanced throughout hematoma as well as in the perihematoma areas. Notably, microglia were present only in the perihematoma areas. Importantly, microglia-specific deletion of CBS impaired spontaneous hematoma resolution following ICH. These results suggested that H$_2$S produced from adjacent cells likely had no effect on microglial phagocytosis. Indeed, SQR, the enzyme initiating irreversible oxidation of H$_2$S, has extremely high H$_2$S oxidation activity [44]. Thus, it has long been assumed that endogenous H$_2$S cannot be maintained at steady concentrations to elicit cellular signaling [44]. In contrast, our recent finding [20] and the results presented here suggested H$_2$S signals through SQR. However, due to the high activity of SQR, endogenous H$_2$S likely functions as an intracellular signaling molecule.

Our results revealed an unexpected role of mitochondrial complex I in regulating microglial phagocytosis. Under certain thermodynamic conditions, such as high $\Delta$Ψ$_m$, electrons are driven back into complex I and consequently reduce oxygen to generate ROS, a phenomenon known as complex I RET [38]. Complex I RET-derived mitochondrial ROS contribute to cellular signaling [45]. Consistent with our recent findings about exogenous H$_2$S, we found that CBS-derived endogenous H$_2$S was oxidized by SQR to repurpose mitochondrial function to ROS production by initiating complex I RET. Moreover, complex I-derived ROS are essential for continual erythropagocytosis by microglia and hematoma resolution following ICH.

Physiologically, elevated expression of UCP2 in peripheral phagocytes promotes continual phagocytosis by reducing $\Delta$Ψ$_m$. Here, we uncovered a new mechanism through which UCP2 activity rather than UCP2 expression was enhanced to promote continual phagocytosis
Fig. 7. Targeting the CBS-H\textsubscript{2}S axis accelerates hematoma resolution in mice with hyperhomocysteinemia. (A and B) Western blot images and quantification data showed that hyperhomocysteinemia abolished the elevation of CBS expression in the hemorrhagic striatum at day 3 following autologous blood-induced ICH (bICH, 3 independent replicates). Hyperhomocysteinemia was induced by feeding mice methionine (Meth), and mice fed normal water served as controls (Nor). (C and D) Hyperhomocysteinemia delayed spontaneous hematoma resolution in mice, and the H\textsubscript{2}S donor ADT accelerated hematoma clearance in mice with hyperhomocysteinemia, as indicated by hemorrhagic volumes at days 5 and 14 following bICH (n = 6). VEH: vehicle. (E-G) Hyperhomocysteinemia delayed functional recovery following bICH, and ADT accelerated functional recovery in mice with hyperhomocysteinemia (E: neuroscores; F: forelimb placement test; G: corner test; n = 6).
following ICH. Indeed, cytosolic ROS generated by NADPH oxidase have been reported to promote the maturation of efferosomes in macrophages [46]. We provided additional clues about how mitochondrial ROS promoted phagocytosis by activating UCP2.

Despite many endeavors to identify therapeutic targets for ICH, no drug is currently available for treating ICH. Augmenting endogenous hematoma resolution is considered a promising strategy for treating ICH [12,14]. However, how erythrocytes are spontaneously and continually cleared from the hemorrhagic parenchyma remains unclear. For the first time, we showed that the microglial CBS-H$_2$S-complex I axis was critical to spontaneous hematoma clearance following ICH. Notably, CBS in peripheral myeloid phagocytes contributed little to spontaneous hematoma resolution. Consistently, by using bone marrow chimeric mice, a recent publication showed that clearance of RBCs following subarachnoid hemorrhage was mainly achieved by brain-resident microglia [22].

In this study, we found that hyperhomocysteinemia exacerbated ICH injuries by inhibiting ICH-induced CBS expression to delay spontaneous hematoma clearance following ICH, while exogenous supplementation with H$_2$S accelerated hematoma resolution following hyperhomocysteinemia-associated ICH. It has been reported that hyperhomocysteinemia inhibits CBS expression through hypermethylation of the CBS promoter [47]. It is possible that hyperhomocysteinemia suppressed ICH-enhanced CBS expression through the same mechanism. Notably, when CBS is less active, homocysteine substitutes cysteine, becoming a significant source of H$_2$S under hyperhomocysteinemia [48]. However, previous research showed that CSE was not expressed by microglia [49]. More importantly, our results showed that CSE was not expressed in microglia in the hemorrhagic striatum following ICH (Supplementary Fig. 2). These results suggested that hyperhomocysteinemia was unlikely to act through CSE to modulate microglial phagocytosis of erythrocytes under hemorrhagic conditions. Collectively, our results suggested that hyperhomocysteinemia delayed hematoma resolution, possibly by inhibiting the CBS-H$_2$S axis following ICH.

There are some limitations of the study. Elevated uncoupling activity of UCP2 in phagocytes promotes continual clearance of dead cells [4]. Notably, UCP2 displays a weak uncoupling activity unless it is activated by endogenous factors, such as ROS [36]. Here, we provided evidence that activation of UCP2 by mitochondrial ROS was likely the mechanism through which ICH-enhanced expression of CBS facilitated clearance of RBCs and hematoma resolution following ICH. However, the comprehensive molecular mechanisms underlying how CBS, ROS generation and mitochondria are involved in promoting microglial phagocytosis of erythrocytes and hematoma resolution following ICH warrant further investigation. We have reported that SQR initiates the generation of mitochondrial ROS via complex I RET by oxidizing sulfides [20]. Here, we showed that CBS and SQR were indispensable for enhanced ROS generation in microglia treated with RBC lysate, suggesting that sulfide production and oxidation were essential for mitochondrial ROS generation induced by RBC lysate. However, we cannot exclude the possibility that other signaling pathways are also involved in redox responses and mitochondrial activation induced by RBC lysate, and this warrants further investigation. CBS is the major H$_2$S synthase in the brain. In addition, inorganic H$_2$S donors have been reported to induce ROS generation in cells in an SQR-dependent manner [50]. This suggested that H$_2$S derived from CBS may be a possible candidate that was oxidized by SQR to induce ROS generation, which was required for the activation of UCP2. Although we used ZnCl$_2$ as an H$_2$S scavenger and found that ZnCl$_2$ blocked the promoting effects of CBS on ROS generation and microglial phagocytosis of RBCs, we cannot exclude other effects of zinc. On the other hand, oxidation of H$_2$S also leads to the formation of polysulfides. Moreover, although ADT and its in vivo active metabolite ADT-OH are widely used as H$_2$S donors that release H$_2$S in the presence of enzymes [34], they are also polysulfides. Indeed, polysulfides emerge as signaling molecules on their own [51]. Thus, further investigation is needed to validate that H$_2$S is the bioactive sulfide derived from CBS that promotes hematoma resolution and to explore whether other sulfide metabolites are also involved. Finally, we showed that hyperhomocysteinemia suppressed ICH-enhanced CBS expression and delayed hematoma resolution following ICH, while supplementation with the organic sulfide ADT accelerated hematoma resolution. However, more experiments are needed to explore how hyperhomocysteinemia retards hematoma resolution by modulating the metabolic activity of CBS and other proteins.

To conclude, this study provides evidence that the CBS-H$_2$S-complex I axis is critical to continual phagocytosis following ICH. Moreover, our results indicate that targeting the CBS-H$_2$S axis may be a promising approach to treat ICH patients with hyperhomocysteinemia.

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Declaration of competing interest
None.

Data availability
Data will be made available on request.

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Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2022.102442.

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