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Downregulation of cystathionine β-synthase/hydrogen sulfide contributes to rotenone-induced microglia polarization toward M1 type

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ABSTRACT

Microglia-mediated neuroinflammation is implicated in the pathogenesis of several neurodegenerative disorders. Microglia can be activated and polarized to exert pro- or anti-inflammatory roles in response to specific stimulus. Rotenone is an environmental toxin that has been shown to activate microglia and neuroinflammation. However, the effects and mechanisms of rotenone on microglia polarization are poorly studied. In the present study, we demonstrated that rotenone enhanced the levels of M1 phenotypic genes including TNF-α, iNOS and COX-2/PGE_2 but reduced that of M2 markers such as Ym1/2 and IL-10 in mouse primary and immortalized microglia. Moreover, the transcription and protein expression of cystathionine-β-synthase (CBS), as well as hydrogen sulfide (H_2S) production were decreased in rotenone-treated primary microglia. Elevating endogenous H_2S via CBS over-expression in immortalized microglia not only reduced the expression of pro-inflammatory M1 genes, but also enhanced the anti-inflammatory M2 marker IL-10 production in response to rotenone stimulation as compared to vector-transfected cells. Similarly, pretreatment with H_2S donor NaHS (50, 100 and 500 μmol/L) attenuated the increases of M1 gene expression triggered by rotenone treatment, and enhanced the M2 gene Ym1/2 expression in mouse primary microglia. In addition, we observed reactive oxygen species (ROS) scavenger N-acetyl-L-cysteine reversed the down-regulation of CBS and H_2S generation caused by rotenone in microglia. NaHS pretreatment also decreased the ROS formation in rotenone-stimulated microglia. Taken together, these results reveal that probably via triggering ROS formation, rotenone suppressed the CBS–H_2S pathway and thus promoted microglia polarization toward M1 pro-inflammatory phenotype.

1. Introduction

Hydrogen sulfide (H_2S) emerges as a novel gasotransmitter although it has been known as a toxic gas for many decades. It can be produced from cysteine and homocysteine by the enzymes including cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (3-MST) [1]. A great amount of H_2S has been detected in the brain [2,3], although its exact concentration range is still in debate due to the limitation of various detection methods. H_2S regulates a variety of physiological processes in the brain, such as facilitating long-term potentiation in the hippocampus [3], and regulating intracellular Ca^{2+} and pH in neuron and glial cells [4–7]. More importantly, H_2S confers neuroprotection against both acute and chronic progression in models of neurological diseases [8,9], at least in part by its inhibition on neuroinflammation, which is mainly mediated by microglia, the resident macrophage in brain. Like macrophage, microglial cells are highly plastic and driven to adopt diverse phenotypes in response to specific stimuli. Classically activated microglia (also referred to as M1-type polarized microglia) are featured by pronounced generation of pro-inflammatory mediators while alternatively activated microglia (M2) are characterized by increased production of anti-inflammatory factors. Functionally, M1 phenotypic microglia are neurotoxic whereas M2 microglia are neuroprotective [10,11].

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Rotenone is a natural pesticide commonly used in farming and fishing, and is now recognized as an environmental toxin for Parkinson's disease (PD). Rotenone is able to activate microglia and trigger neuroinflammatory responses, which in turn exacerbates its neurotoxicity to dopaminergic neurons [12,13]. The microglial activation, associated with accumulation of pro-inflammatory mediators, has been well described in rotenone-intoxicated PD models [14].

Our previous studies demonstrated that sodium hydrosulfide (NaHS, an exogenous H_2S source) inhibited nitric oxide and TNF-α production in the striatum of rotenone-induced rat models of PD and produced protective action on dopaminergic neurons in midbrain [9]. In a recent study, we showed H_2S promoted M2 polarization and suppressed neuroinflammation in lipopolysaccharide-stimulated microglia [15]. However, whether rotenone affects microglial polarization and whether it involves H_2S pathway alteration remain unclear. Therefore, the present study was sought to address this issue.

2. Materials and methods

2.1. Chemicals and reagents

NaHS, rotenone, and N-acetyl-L-cysteine (NAC) were purchased from Sigma–Aldrich (St. Louis, MO, USA). The sources for primary antibodies were listed as follows: anti-Iba-1 (Abcam, ab5076, Hong Kong, China); anti-CBS (sc-67154) and anti-cyclooxygenase-2 (COX-2) (Santa Cruz, CA, USA); anti-CBS (Abnova H00000875-M01, Taipei, Taiwan); anti-β-actin (sigma–Aldrich, A3854, St. Louis, MO, USA). Reagents for cell culture were bought from Gibco (Grand Island, USA).

2.2. Cell culture and treatment

Primary microglia cultures were prepared from 1-day-old postnatal C57BL/6J mice. Briefly, the cortex was minced and dissociated in 0.125% Trypsin for 4 min at 37 °C. Trypsin was then neutralized with complete media [Dulbecco modified Eagle's medium (DMEM)/F12 supplemented with 10% heat-inactivated fetal bovine serum (FBS)] and strained through a 200 μm mesh filter. After brief centrifugation, cells were harvested and plated in T75 cell culture flasks. The medium was replaced every 3 days. Once the lower layer of astrocytes reached confluence, microglial cells were harvested by mechanical agitation at 180 rpm for 70 min and subsequently plated in DMEM/F12 supplemented with 10% FBS at a desired density for further experimentation. The purity of microglia reached 90% in our study. The experimental procedures were approved by the Institutional Animal Care and Use Committee of Soochow University.

Murine microglial BV2 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin in an incubator with 95% air/5% CO_2 at 37 °C. Cells were regularly sub-cultured three times a week and seeded into 35 mm dishes or 24-well plates prior to treatment.

2.3. Quantitative and reverse transcription PCR

Quantitative PCR was performed as we previously described [15] with the primers listed as follows: mouse iNOS (forward: 5′-CAGGAGGAGAGAGATCCGATTTA-3′, reverse: 5′-GCATTAGCATGGAAGCAAAGA-3′); mouse TNF-α (forward: 5′-CATCTTCTCAAAATTCGAGTGACAA-3′, reverse: 5′-TGGGAGTAGACAAGGTACAACCC-3′); mouse Ym1/2 (forward: 5′-CAGGGTAATGAGTGGGTTGG-3′, reverse: 5′-CACGGCACCTCCTAAATTGT-3′); and mouse 18S (forward: 5′-TCAACACGGGAAACCTCAC-3′, reverse: 5′-CGCTCCACCAACTAAGAAC-3′). 18S RNA was used as an internal control. The results were normalized and expressed as ratios of the target gene over 18S. The mRNA levels of COX-2 and β-actin were determined by two-step reverse transcription PCR with primers as listed: mouse COX-2 (sense 5′-CAGCAAATCCTTGCTGTT-3′; antisense 5′-TGGGCAAAGAATGCAAACATC-3′); mouse β-actin (sense 5′-GACTACCTCATGAAGATCCT-3′; antisense 5′-CCACATCTGCTGGAAGGTGG-3′).

2.4. Immunocytochemistry

Cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% saponin in phosphate buffered saline (PBS), followed by blocking with PBS containing 10% bovine serum albumin. Next, cells were incubated with goat anti-Iba-1 (1:200) and rabbit anti-CBS (1:200) at 4 °C overnight. After that, cells were briefly washed and incubated with Alexa Fluor® 555 donkey anti-goat IgG and Alexa Fluor® 488 donkey anti-rabbit IgG in dark for 1 h. Finally, coverslips were mounted with the mounting medium containing DAPI (Vector Laboratories H-1200, Burlingame, CA, USA). Images were taken under the fluorescent microscope (Zeiss, AXIO Scope A1, Goettingen, Germany).

2.5. Western blot analysis

Cells were washed with PBS and lysed in ice-cold lysis buffer (150 mM NaCl, 25 mM Tris, 5 mM EDTA, 1% Nonidet P-40, pH 7.5) with protease inhibitor cocktail tablets (Roche Diagnostics, Penzberg, Germany). Protein concentration was determined by BCA protein assay kit (Pierce, Rockford, IL, USA). Protein samples were separated by 10% sodium dodecyl sulfate–polyacrylamide gels and transferred onto nitrocellulose membranes. Next, membranes were blocked in 5% (w/v) non-fat dry milk powder in 0.1% Tris buffered saline/Tween 20 (TBST) for 1 h and incubated with primary antibodies at optimized dilutions at 4 °C overnight. After that, membranes were washed with TBST and incubated with HRP-conjugated secondary antibodies for 1 h. Membranes were finally visualized using ECL chemiluminescence (Thermo Company, West Chester, PA, USA). The band densities were analyzed with Image J software (National Institute of Health, USA).

2.6. H_2S measurement

H_2S production, which was indicated by the sulfide level in culture supernatant, was determined as we previously reported [16]. Briefly, 400 μl medium were collected, in which 4 μl of 1 M NaOH was added and gently mixed, followed by the addition of 40 μl of 20 mM N,N-dimethyl-p-phenylenediamine sulfate in 7.2 M HCl and 40 μl of 30 mM $FeCl_3$ in 1.2 M HCl. The reaction mixture was left at room temperature for 20 min. The formed methylene blue was detected at 668 nm using the microplate reader (Infinite M200 PRO, Grodig, Austria). The H_2S concentration was assessed by a standard curve of NaHS (0–100 μM).

2.7. CBS overexpression

BV2 cells were transiently transfected with CBS-pME18S-HA plasmid (a gift from Dr. Hideo Kimura) or its vector using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The CBS gene was subcloned from the sequence as previously published [17]. It is inserted into the EcoR1 and Not I sites of pME18S under the transcriptional control of SR alpha promoter. 24 h after transfection, cells were washed once with PBS and subjected to rotenone treatment for different time periods as specified in text.

2.8. Prostaglandin E_2 (PGE_2) and IL-10 assay

The PGE_2 assay was performed at 48 h after rotenone treatment (10 nM) while IL-10 was determined at 24 h after treatment, based on our preliminary study. In brief, the cell-free culture supernatant was collected, in which the PGE_2 and IL-10 contents were determined with ELISA kits from Thermo Scientific and BOSTER (Wuhan, China), respectively. The absorbance was determined at 450 nm by the microplate reader as described above.

2.9. Intracellular reactive oxygen species (ROS) assay

Intracellular ROS was evaluated using the DCFH-DA probe (Sigma–Aldrich, D6883, St. Louis, MO, USA). In brief, cells were loaded with DCFH-DA (10 μM at a final concentration) in PBS for 45 min at 37 °C in dark. After rinsing twice with chilled PBS solution, the fluorescence was either read by the aforementioned microplate reader at an excitation wavelength (Ex) of 490 nm with an emission wavelength (Em) of 520 nm or visualized under an inverted fluorescent microscope. The value was normalized to that of control group.

2.10. Statistical analysis

All data are presented as mean ± SEM. Statistical significance was assessed by student *t* test for two-group comparison or one-way analysis of variance followed by a post hoc (Tukey) test for multiple group comparison. Differences with *P* value <0.05 were considered statistically significant.

3. Results

3.1. Rotenone suppressed CBS expression and H_2S generation in mouse primary microglia

CBS is primarily responsible for H_2S synthesis in the brain and highly expressed in astrocytes [18]. In this study, we observed that CBS protein obviously co-localized with the microglial marker Iba-1 in primary microglia enriched culture from neonatal mice (Fig. 1A), indicating that CBS is also abundant in mouse microglia. Moreover, microglial CBS mRNA and its protein expression decreased approximately by 66% and 36% following 10 nmol/L rotenone treatment for 12 h and 24 h, respectively, when compared with vehicle group (Fig. 1B and C). In addition, we found that the sulfide level in culture supernatant dropped from 5.66 ± 0.09 μmol/L to 3.48 ± 0.16 μmol/L when microglial cells were treated with rotenone for 48 h (Fig. 1D). These results indicate that rotenone may suppress CBS transcription and thus impair endogenous H_2S generation in microglia.

CBS overexpression elevated H_2S production and promoted the polarization transition from M1 to M2 phenotype in rotenone-treated microglia

In our previous study, we demonstrated that 10 nmol/L rotenone was sufficient to activate microglia associated with NF-kB activation [9]. To explore if enhancing endogenous H_2S production could affect microglial polarization, CBS was transiently transfection into immortalized murine microglia (BV2 cells). As shown in Fig. 2A and B, transfection with CBS-pME18S-HA plasmid resulted in a significant elevation of CBS protein expression and sulfide level in the culture supernatant, as compared to non-transfected (control) or vector-transfected BV2 cells. Notably, rotenone triggered the polarization toward M1 phenotype in immortalized microglia, as evidenced by a marked increase in pro-inflammatory mediators

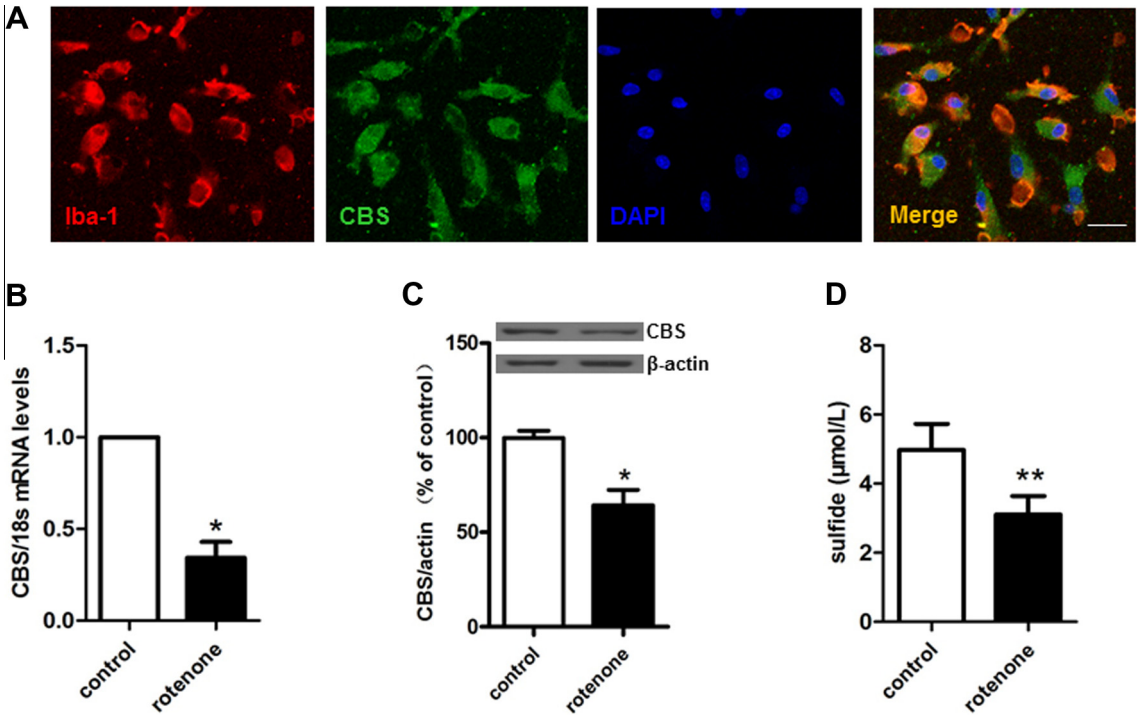

Fig. 1. Rotenone suppressed CBS expression and H_2S production in primary microglia. (A) Immunofluorescent staining revealed that CBS expressed and co-localized with Iba-1 positive cells in primary microglia-enriched cultures from neonatal mice. Scale bar: 20 μm. (B–D) CBS mRNA (B) and protein expression (C), as well as H_2S production (D) were reduced in rotenone (10 nM)-stimulated primary microglia. The mRNA level was determined by real-time PCR and normalized to that of 18S as internal control. β-Actin served as loading controls. Mean ± SEM, $N = 3$. Student *t* test was used for two-group comparison. $^{*}P < 0.05$, $^{***}P < 0.001$ versus controls.

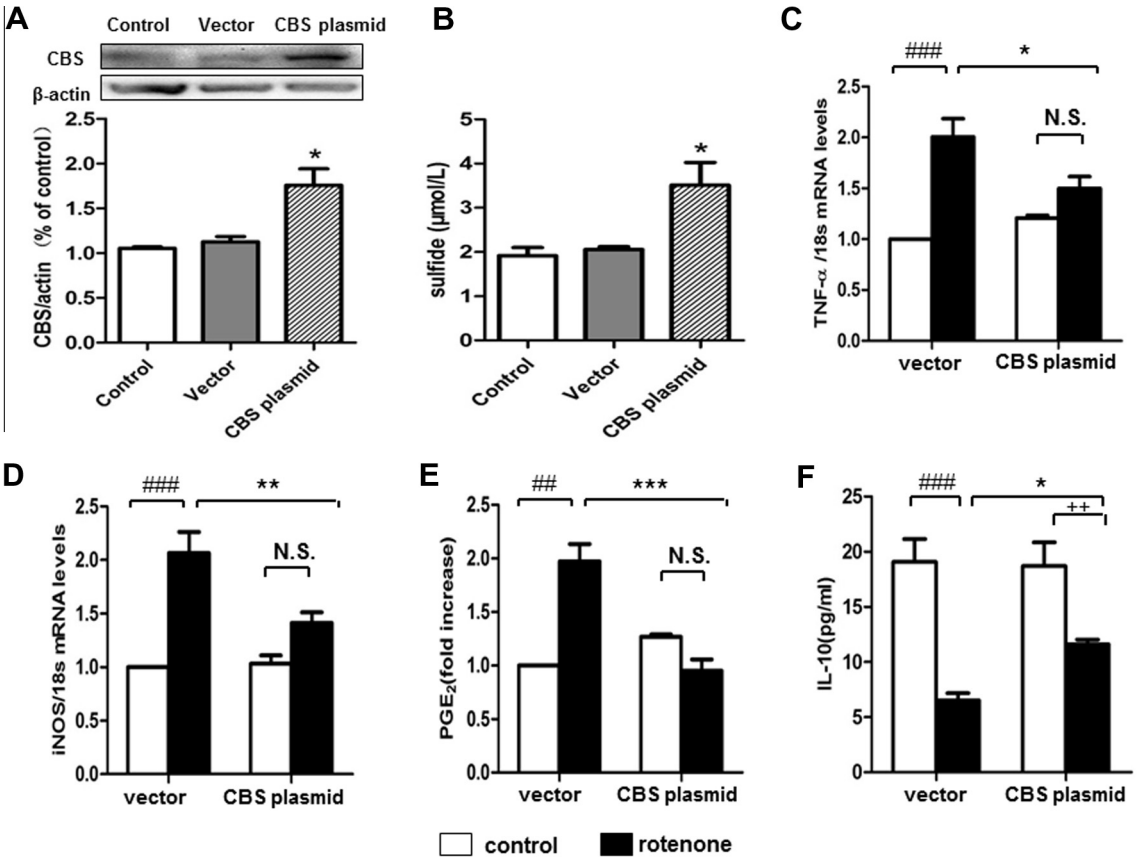

Fig. 2. CBS overexpression elevated H_2S production and promoted microglial polarization transition from M1 toward M2 phenotype. Immortalized microglial cells (BV2) were transiently transfected with CBS-pME18S-HA plasmid or vector, followed by rotenone treatment. (A and B) CBS protein level and H_2S production was enhanced after CBS transfection. (C–F) The changes of TNF-α (C) and iNOS (D) mRNA, PGE_2 (E) and IL-10 (F) levels in vehicle or rotenone-treated BV2 cells following transfection, as determined by quantitative PCR and ELISA, respectively. Data were presented as mean ± SEM, $n = 3–8$. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus vehicle-treated vector group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus rotenone-treated vector group. $^{++}P < 0.01$ versus CBS transfected group, N.S., not significant.

including TNF-α, iNOS and PGE_2, accompanied by a significant decrease of M2 marker IL-10 in vector transfected cells following rotenone (10 nmol/L) treatment. However, CBS transfection markedly reduced the mRNA levels of pro-inflammatory M1 genes TNF-α (Fig. 2C) and iNOS (Fig. 2D) as compared to vector-transfected BV2 cells following rotenone exposure. The stimulatory effect of rotenone on the production of pro-inflammatory factor PGE_2 was also blocked in CBS-transfected cells (Fig. 2E). Moreover, CBS overexpression enhanced the anti-inflammatory M2 marker IL-10 production in rotenone-treated cells (Fig. 2F). These data imply that elevating endogenous H_2S production via CBS over-expression could promote the rotenone-treated microglial polarization from M1 toward M2 phenotype.

3.2. NaHS promoted the M2 polarization of rotenone-stimulated microglia

We further investigated if exogenous H_2S application with NaHS also affected the microglial polarization in mouse primary microglia. As shown in Fig. 3 and 10 nmol/L rotenone treatment for 12 h evoked the M1 polarization of primary microglia, as evidenced by a marked increase in the mRNA and protein levels of pro-inflammatory M1 genes (TNF-α and iNOS mRNA, COX-2/PGE_2 protein) and a slight decrease of M2 gene Ym1/2, without altering the cell viability (data not shown). This is consistent with our observations in immortalized murine microglia (Fig. 2). NaHS pretreatment (50, 100 and 500 μmol/L) for 10 min not only reduced the increases of M1 gene expression (TNF-α and iNOS mRNA) triggered by rotenone, but also enhanced the M2 gene Ym 1/2 expression. However, the dose-dependent effect of NaHS on the gene expression was not obtained. 100 μmol/L NaHS produced the maximal effect on these polarization markers. NaHS at 500 μmol/L yielded less significant effects. In addition, we observed that 100 μmol/L NaHS pretreatment also attenuated the increase in COX-2 mRNA and protein expression, and diminished the PGE_2 production in rotenone-treated microglial cells (Fig. 3D–F).

3.3. ROS was involved in CBS down-regulation in rotenone-stimulated microglia

Several studies reveal that ROS are essential for rotenone-stimulated microglial activation and the subsequent neurotoxicity to midbrain neurons [13]. To test if ROS contributed to the decrease of CBS expression and H_2S generation in microglia, we examined CBS protein and sulfide level in the presence or absence of ROS scavenger NAC following rotenone stimulation. As can be seen from Fig. 4A and B, rotenone treatment resulted in a remarkable increase of ROS generation in immortalized murine microglial cells, as evidence by strong DCFH fluorescence in rotenone-treated group (Fig. 4A-b) compared to vehicle-treated group (Fig. 4A-a). The rotenone-induced fluorescence was substantially reduced by NAC (5 mmol/L) co-treatment, implying NAC scavenged the intracellular ROS. Moreover, co-treatment with NAC reversed the decrease of CBS expression (Fig. 4C) and H_2S generation (Fig. 4D)

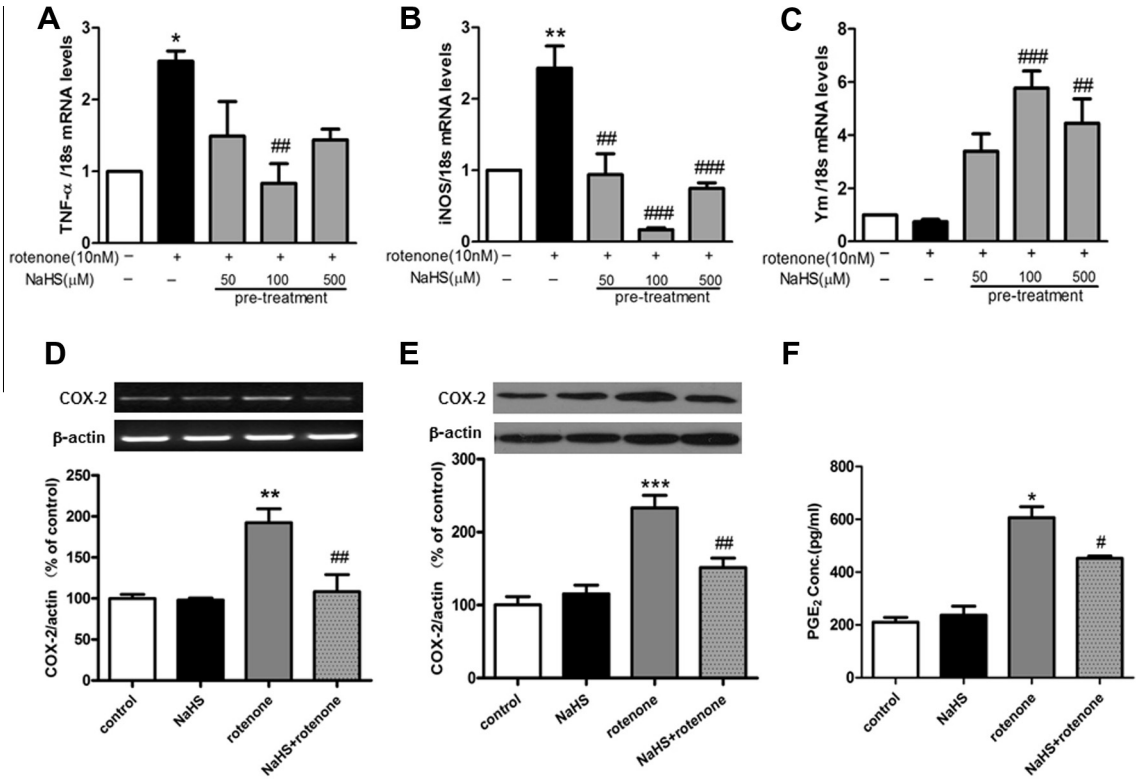

Fig. 3. NaHS promoted M2 polarization of rotenone-stimulated microglia. Cells were pretreated with NaHS at indicated concentration for 10 min, followed by rotenone treatment. (A–C) Quantitative PCR results showed the mRNA levels of TNF-α (A), iNOS (B) and Ym1/2 genes (C) in mouse primary microglia at 12 h after rotenone stimulation. Results were normalized by 18S and expressed as the relative value to controls. (D–F) Cells were pretreated with 100 μM NaHS for 10 min before 10 nM rotenone treatment. COX-2 mRNA (D), protein (E) and PGE_2 (F) levels were determined at 12 h, 24 h and 48 h after rotenone treatment with reverse transcription PCR, Western blotting and ELISA, respectively. Mean ± SEM, $n = 4$. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus controls; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus rotenone group.

induced by rotenone in mouse primary microglia, although NAC alone did not significantly affect CBS expression or sulfide level. In addition, we observed that application with NaHS (100 μmol/L) produced similar effects to NAC. NaHS pre-treatment also significantly blocked the increase of ROS generation in rotenone-stimulated microglia. These findings suggest that the rotenone-stimulated burst of ROS generation may be involved in CBS suppression and thus H_2S production decrease, and also imply that H_2S may produce a feedback inhibition on ROS formation.

4. Discussion

Increasing evidence indicates that like macrophage, microglia represent heterogeneous cell populations with functional variability due to the differences in polarization status, which may be stimulus-dependent. M1 polarized microglia are pro-inflammatory while M2 phenotype are anti-inflammatory. In the present study, we demonstrated that rotenone suppressed the CBS–H_2S pathway and evoked M1-like polarization in mouse primary and immortalized microglia. Stimulation of endogenous H_2S via CBS overexpression or supplementation with NaHS inhibited the M1 polarization and promoted the rotenone-treated microglia toward M2 phenotype transition. The results indicate that the suppression of CBS expression and H_2S generation may be involved in rotenone-treated microglia polarization toward M1 phenotype and thus represent as pro-inflammatory.

The concept of microglial phenotypic transition emerged into the field of PD research in recent years. Both M1 and M2 markers have been detected in the serum and cerebrospinal fluid of PD patients [19,20]. However, the cellular and molecular mechanisms remain elusive. In this study, we showed that rotenone increased the mRNA and protein levels of pro-inflammatory factors (TNF-α, iNOS and COX-2/PGE_2) but decreased that of anti-inflammatory markers (Ym1/2 and IL-10) in both mouse primary and immortalized microglia. This indicates that rotenone evokes microglia polarization toward M1-like phenotype, which is in line with our previous reports that the pro-inflammatory cytokine/factors such as TNF-α and nitric oxide were elevated in the striatum of rotenone-induced parkinsonian rodent [9].

The signaling molecule that guide or modulate microglia polarization is a hot topic of neuroinflammation research. Our study showed that both exogenous and endogenous H_2S promoted the polarization transition from M1 to M2 phenotype in rotenone-treated microglia. In a recent study, we reported that H_2S activated adenosine monophosphate activated protein kinase (AMPK), by which H_2S promoted M2 polarization of microglia and thereby suppressed lipopolysaccharide-stimulated neuroinflammation [15]. This led us to propose that a similar mechanism may be involved in eliciting rotenone-treated microglial polarization transition. H_2S serves as a physiologically relevant gasotransmitter in maintaining homeostasis probably via S-sulfhydrated modification. Therefore, the candidate protein that undergoes S-sulfhydration and drives microglial polarization transition deserves further investigation.

In this study we identified CBS expression and its co-localization with microglial marker Iba-1 in primary microglia-enriched culture. CBS is abundantly expressed and most relevant for H_2S biosynthesis in the brain, although 3-MST and CSE also exist [21,22]. CBS is highly expressed in glia and may thus be responsible for H_2S modulation during inflammation. Interestingly, the mRNA expression of CSE rather than CBS was detected in primary microglia isolated from neonatal rats [23]. In this study, we verified the expression of CBS in mouse primary microglia with different

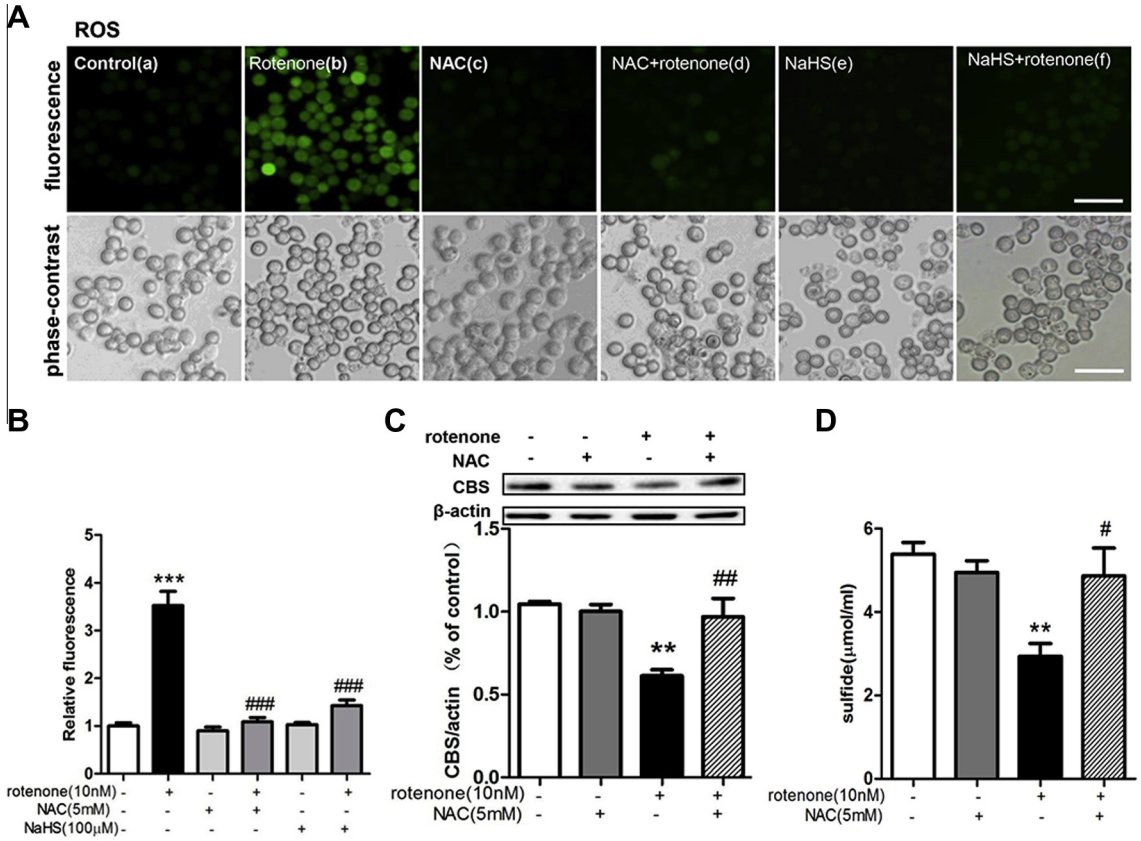

Fig. 4. ROS was involved in CBS down-regulation in rotenone-stimulated microglia. (A and B) Pretreatment with NAC or NaHS diminished the ROS formation at 18 h after 10 nM rotenone exposure in immortalized microglia. ROS was visualized with a fluorescent probe DCFH-DA under a Zeiss inverted fluorescent microscope. Scale bar: 50 μm. (C and D) ROS scavenger NAC reversed the decrease of CBS expression and H_2S production caused by rotenone treatment for 48 h in mouse primary microglia. Mean ± SEM. $N = 3–5$. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus rotenone group.

approaches. Apart from immunofluorescent staining, quantitative PCR and western blot analysis displayed the existence of CBS in mouse primary microglia. In addition, we used two different commercially available antibodies against mouse CBS (Santa Cruz, sc-67154; Abnova, H00000875-M01) to verify our results. The reason for the differential expression pattern of H_2S biosynthase in these two studies is unknown, probably due to the difference in species.

Although the anti-neuroinflammatory role of H_2S has been well documented, the microglial endogenous H_2S in response to inflammatory stimuli and its consequences were poorly studied. Our data displayed that rotenone decreased the mRNA and protein levels of CBS and reduced H_2S production in primary microglia. Elevation of H_2S elicited an anti-inflammatory action. It implies that suppression of CBS–H_2S pathway may be involved in rotenone-induced microglia polarization to M1 phenotype. CBS is a rate-limiting enzyme in the trans-sulfuration pathway and involved in the removal of homocysteine from the cycle and the biosynthesis of cysteine, which is a precursor of glutathione [24]. Its activity is highly regulated in mammals and could be enhance by S-adenosyl-L-methionine. Carbon monoxide and nitric oxide has been shown to modulate CBS activity by binding to its heme center. Our current data displayed that ROS scavenger NAC was able to reverse the rotenone-induced reduction of CBS expression and H_2S production in microglia, implying that ROS may serve as a suppressor of CBS expression during redox stress. Our findings supported the notion that ROS are critical for rotenone-induced microglia activation and thus inflammation in the brain. It may also be relevant in explaining ROS-induced depletion of glutathione in brain disorders. However, the basal levels of ROS may not be sufficient in suppressing CBS–H_2S pathway in normal situations as NAC treatment alone did not significantly modulate CBS expression or H_2S generation. By contrast, the buildup of potentially damaging levels of ROS due to pathologic stimulations may lower CBS expression via unidentified mechanisms. In addition, we observed that NaHS pretreatment blocked the ROS accumulation in rotenone-stimulated microglia. This indicates that H_2S may yield a negative feedback on ROS formation, preventing an overwhelming suppression by rotenone on CBS–H_2S pathway.

In sum, our study demonstrated that rotenone suppressed CBS expression dependent of intracellular ROS formation and thereby impaired endogenous production of H_2S, which plays an important role in directing microglia polarization toward M2 anti-inflammatory phenotype.

Conflict of interest

The authors have no conflict of interest.

Acknowledgments

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References

[1] L.F. Hu, M. Lu, P.T. Hon Wong, J.S. Bian, Hydrogen sulfide: neurophysiology and neuropathology, Antioxid. Redox Signal. 15 (2011) 405–419.

[2] L.R. Goodwin, D. Francom, F.P. Dieken, J.D. Taylor, M.W. Warenycia, R.J. Reiffenstein, G. Dowling, Determination of sulfide in brain tissue by gas dialysis/ion chromatography: postmortem studies and two case reports, J. Anal. Toxicol. 13 (1989) 105–109.

[3] K. Abe, H. Kimura, The possible role of hydrogen sulfide as an endogenous neuromodulator, J. Neurosci. 16 (1996) 1066–1071.

[4] Y. Nagai, M. Tsugane, J. Oka, H. Kimura, Hydrogen sulfide induces calcium waves in astrocytes, FASEB J. 18 (2004) 557–559.

[5] S.W. Lee, Y.S. Hu, L.F. Hu, Q. Lu, G.S. Dawe, P.K. Moore, P.T. Wong, J.S. Bian, Hydrogen sulphide regulates calcium homeostasis in microglial cells, Glia 54 (2006) 116–124.

[6] Q.C. Yong, C.H. Choo, B.H. Tan, C.M. Low, J.S. Bian, Effect of hydrogen sulfide on intracellular calcium homeostasis in neuronal cells, Neurochem. Int. 56 (2010) 508–515.

[7] M. Lu, C.H. Choo, L.F. Hu, B.H. Tan, G. Hu, J.S. Bian, Hydrogen sulfide regulates intracellular pH in rat primary cultured glia cells, Neurosci. Res. 66 (2010) 92–98.

[8] J. Yin, C. Tu, J. Zhao, D. Ou, G. Chen, Y. Liu, X. Xiao, Exogenous hydrogen sulfide protects against global cerebral ischemia/reperfusion injury via its anti-oxidative, anti-inflammatory and anti-apoptotic effects in rats, Brain Res. 1491 (2013) 188–196.

[9] L.F. Hu, M. Lu, C.X. Tiong, G.S. Dawe, G. Hu, J.S. Bian, Neuroprotective effects of hydrogen sulfide on Parkinson's disease rat models, Aging Cell 9 (2010) 135–146.

[10] K.A. Kigerl, J.C. Gensel, D.P. Ankeny, J.K. Alexander, D.J. Donnelly, P.G. Popovich, Identification of two distinct macrophage subsets with divergent effects causing either neurotoxicity or regeneration in the injured mouse spinal cord, J. Neurosci. 29 (2009) 13435–13444.

[11] X. Hu, P. Li, Y. Guo, H. Wang, R.K. Leak, S. Chen, Y. Gao, J. Chen, Microglia/macrophage polarization dynamics reveal novel mechanism of injury expansion after focal cerebral ischemia, Stroke 43 (2012) 3063–3070.

[12] H.M. Gao, J.S. Hong, W. Zhang, B. Liu, Distinct role for microglia in rotenone-induced degeneration of dopaminergic neurons, J. Neurosci. 22 (2002) 782–790.

[13] H.M. Gao, B. Liu, J.S. Hong, Critical role for microglial NADPH oxidase in rotenone-induced degeneration of dopaminergic neurons, J. Neurosci. 23 (2003) 6181–6187.

[14] T.B. Sherer, R. Betarbet, J.H. Kim, J.T. Greenamyre, Selective microglial activation in the rat rotenone model of Parkinson's disease, Neurosci. Lett. 341 (2003) 87–90.

[15] X. Zhou, Y. Cao, G. Ao, L. Hu, H. Liu, J. Wu, X. Wang, M. Jin, S. Zheng, X. Zhen, N.J. Alkayed, J. Jia, J. Cheng, CaMKKbeta-dependent activation of AMP-activated protein kinase is critical to suppressive effects of hydrogen sulfide on neuroinflammation, Antioxid. Redox Signal. (2014).

[16] X.H. Wang, F. Wang, S.J. You, Y.J. Cao, L.D. Cao, Q. Han, C.F. Liu, L.F. Hu, Dysregulation of cystathionine gamma-lyase (CSE)/hydrogen sulfide pathway contributes to ox-LDL-induced inflammation in macrophage, Cell. Signal. 25 (2013) 2255–2262.

[17] K. Eto, H. Kimura, A novel enhancing mechanism for hydrogen sulfide-producing activity of cystathionine beta-synthase, J. Biol. Chem. 277 (2002) 42680–42685.

[18] M. Lee, C. Schwab, S. Yu, E. McGeer, P.L. McGeer, Astrocytes produce the antiinflammatory and neuroprotective agent hydrogen sulfide, Neurobiol. Aging 30 (2009) 1523–1534.

[19] D. Blum-Degen, T. Muller, W. Kuhn, M. Gerlach, H. Przuntek, P. Riederer, Interleukin-1 beta and interleukin-6 are elevated in the cerebrospinal fluid of Alzheimer's and de novo Parkinson's disease patients, Neurosci. Lett. 202 (1995) 17–20.

[20] R.J. Dobbs, A. Charlett, A.G. Purkiss, S.M. Dobbs, C. Weller, D.W. Peterson, Association of circulating TNF-alpha and IL-6 with ageing and parkinsonism, Acta Neurol. Scand. 100 (1999) 34–41.

[21] N. Shibuya, M. Tanaka, M. Yoshida, Y. Ogasawara, T. Togawa, K. Ishii, H. Kimura, 3-Mercaptopyruvate sulfurtransferase produces hydrogen sulfide and bound sulfane sulfur in the brain, Antioxid. Redox Signal. 11 (2009) 703–714.

[22] B.D. Paul, J.I. Sbodio, R. Xu, M.S. Vandiver, J.Y. Cha, A.M. Snowman, S.H. Snyder, Cystathionine gamma-lyase deficiency mediates neurodegeneration in Huntington's disease, Nature 509 (2014) 96–100.

[23] K. Robert, F. Vialard, E. Thiery, K. Toyama, P.M. Sinet, N. Janel, J. London, Expression of the cystathionine beta synthase (CBS) gene during mouse development and immunolocalization in adult brain, J. Histochem. Cytochem. 51 (2003) 363–371.

[24] X. Chen, K.H. Jhee, W.D. Kruger, Production of the neuromodulator H2S by cystathionine beta-synthase via the condensation of cysteine and homocysteine, J. Biol. Chem. 279 (2004) 52082–52086.