A1 Reactive Astrocytes Activated by 2-chloroethanol Modulate M1 Microglia Polarization through Upregulating IL-1 β and TNF- α

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Abstract

Background

1,2-Dichloroethane (1,2-DCE) is a synthetic organic chemical that causes brain edema under subacute poisoning. Our previous studies indicated that the neuroinflammation could be induced due to activation of both astrocytes and microglia during the course of brain edema in 1,2-DCE intoxicated mice. However, the crosstalk between the two glial cells in 1,2-DCE-induced neuroinflammation is unclear. In the current studies, we hypothesized that astrocytes are the first responder to the effects of 1,2-DCE in the brain, as they adhere to the cerebral capillaries, and they are an essential component of the blood-brain barrier (BBB).

Methods

We used primary cultured rat astrocytes and microglia, as well as a highly aggressively proliferating immortalized (HAPI) microglia cell line to study the effects of astrocytes on microglia polarization following exposure to 2-CE.

Results

Findings from the present studies demonstrated that treatment of primary rat astrocytes with 2-chloroethanol (2-CE), the intermediate metabolite of 1,2-DCE *in vivo*, can stimulate the activation of A1 reactive astrocytes (A1s) through p38 mitogen-activated protein kinase (p38 MAPK)/ nuclear factor- κ B (NF- κ B) and activator protein-1 (AP-1) signaling pathways by the reactive oxygen species (ROS) produced during 2-CE metabolism. A1s activated by 2-CE can upregulate the expression of interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α), and inducible nitric oxide synthase (iNOS), and stimulate the M1 polarization of microglia through IL-1 β and TNF- α released by 2-CE activated A1s. Microglia are less sensitive to 2-CE than astrocytes, since treatment of primary rat microglia with 30 mM 2-CE alone failed to activate them, though this dose of 2-CE can activate A1s and in turn stimulate M1 polarization of microglia through the factors released by A1s.

Conclusion

The neuroinflammation induced by 1,2-DCE in the brain of mice is most probably triggered by the activation of astrocytes. The understanding of the multidimensional roles of reactive astrocytes may further the development of new treatment strategies in reducing neuroinflammation and brain edema following 1,2-DCE-induced toxic encephalopathy.

1. Introduction

1,2-Dichloroethane (1,2-DCE) is a synthetic organic chemical that can result in brain edema under subacute poisoning. [1, 2]. The results of our previous studies revealed that the inflammatory reactions mediated by the p38 mitogen-activated protein kinase (p38 MAPK) and nuclear factor- κ B (NF- κ B)

signaling pathways might be involved in matrix metalloproteinase-9 (MMP-9) overexpression, blood-brain barrier (BBB) disruption and edema formation in the brain of 1,2-DCE-intoxicated mice. Moreover, the findings from our *in vitro* studies disclosed that treatment of primary cultured rat astrocytes with 2-chloroethanol (2-CE), the intermediate metabolite of 1,2-DCE via the cytochrome P4502E1 (CYP2E1) *in vivo*, could upregulated MMP-9 expression through the p38 MAPK signaling pathway via activation of both NF- κ B and activator protein-1 (AP-1) [3].

Astrocytes are the most abundant cells in the brain that are necessary for neuronal survival and function by maintenance of BBB integrity and extracellular homeostasis [4]. Accumulated evidences demonstrated that astrocytes can be activated under some pathological conditions, and then secrete a variety of proinflammatory factors that might induce neuroinflammatory responses, which degrades the tight junctions of BBB, thus leading to breakdown in BBB integrity and brain edema formation [5, 6]. However, the functions of activated astrocytes under what contexts they may be helpful or harmful for the injury recovery remain unclear.

Recently, it was reported that neuroinflammation could induce two different types of reactive astrocytes, termed A1 and A2, respectively [7]. The A1 reactive astrocytes (A1s) are harmful since they highly up-regulate many classical complement cascade genes shown to be destructive to neurons. In contrast, A2 reactive astrocytes (A2s) are protective due to up-regulating many neurotrophic factors [8, 9]. Astrocytes, as they adhere to the cerebral capillaries, are an essential component of the BBB [4]. Therefore, astrocytes might be the first exposing cells for the exogenous chemicals in the peripheral circulation from entering the brain. Because of high lipid solubility, 1,2-DCE can easily across the BBB. Thus, it is reasonable to assume that astrocytes might be both the first targets of, as well as the responders to, 1,2-DCE.

Microglia are the primary immune effector cells and play an important role in the response of brain to both exogenous and endogenous insults [10]. As a myeloid-derived cell, activated microglia can polarize into M1 or M2 distinct macrophage subtypes [11]. The M1 phenotype is associated with prototypic inflammatory responses with increased release of proinflammatory cytokines. Adoption of the M2 phenotype promotes tissue repair through release of anti-inflammatory cytokines and neurotrophic factors [12]. Increasing lines of evidence indicate that the crosstalk between microglia and astrocyte play an important role in the neurodegenerative diseases and neurotoxicity of toxicants. A large number of experiments suggested that astrocytes are activated following the microglial activation. However, since astrocytes are the first targets of 1,2-DCE in the brain, and a prominent contributor to several proinflammatory mediators, it is necessary to uncover the alteration in the microglial polarization following the activation of astrocytes. Up-to-date, the important interactions between astrocytes and microglia are virtually unknown in 1,2-DCE induced brain edema.

We previously reported that both astrocytes and microglia are activated during the course of brain edema formation in 1,2-DCE intoxicated mice [13]. In the current study, we postulated that 2-CE directly stimulate astrocytes through p38 MAPK signaling pathway via activation of NF- κ B and AP-1, and that this could in turn enhance microglial polarization. To address this hypothesis, we utilized primary cultured rat

astrocytes and microglia, as well as an immortalized microglia cell line to study the effects of astrocytes on microglia polarization following exposure to 2-CE. Our studies revealed that A1s activated by 2-CE are a potent inducer of M1 polarization in microglia, suggesting the crosstalk between astrocytes and microglia is critical for neuroinflammatory injury from 1,2-DCE poisoning.

2. Materials And Methods

2.1. Reagents

2-Chloroethanol (2-CE) was obtained from Sinopharm Chemical Reagent Co., Ltd. (Ningbo, China), while reagents for the cell culture were purchased from Biological Industries (Beit-Haemek, Israel). The quantitative polymerase chain reaction (qPCR) assay kits were acquired from Takara (Japan). SB202190, pyrrolidine dithiocarbamate (PDTC), SR11302, and N-acetyl-L-cysteine (NAC) were purchased from Selleck (Houston, USA), APExBIO (Houston, USA), or Beyotime Biotechnology (Shanghai, China). Fluorocitrate (FC) and GIBH-130 (GI) were bought from Sigma (New Jersey, USA) and MedChemExpress (New Jersey, USA), respectively. Short hairpin RNA (shRNA) targeting the interleukin-1 β (IL-1 β) gene was designed and synthesized by Hanbio (Shanghai, China), while small interfering RNAs (siRNAs) targeting the tumor necrosis factor- α (TNF- α), inducible nitric oxide synthase (iNOS), or CYP2E1 genes were designed and synthesized by RiboBio (Guangzhou, China).

2.2. Primary cultures of astrocytes and microglia

Astrocytes and microglia were purified from the cerebral cortices of 1–2-day-old postnatal Wistar rats that were obtained from the Animal Experiment Center of China Medical University, which were cultured as previously described [3, 14, 15]. Briefly, the cerebral cortices of rat brains were taken immediately after decapitation, and carefully cleaned of meninges and blood vessels. They were enzymatically and mechanically dissociated to generate a single cell suspension. The cells were then seeded and incubated at 37°C in a 5% CO₂-humidified atmosphere in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The medium was completely replaced every 3 d until the cells reached a confluence, which usually occurred after 12–14 d.

The microglia were isolated from confluent mixed glial cultures via the mild trypsinization [14, 15]. After 0.08% trypsinization, the microglia were firmly attached to the bottom of the culture plates, and the supernatant containing a detached intact astrocyte layer was collected and reseeded on culture plates pre-coated with Poly-L-Lysine. The astrocytes were purified via four passages to remove oligodendrocytes and neurons. By contrast, the microglia were incubated for 24 h with 200 ng/mL macrophage colony-stimulating factor (MCSF, Novoprotein Scientific, Shanghai, China) to stimulate their proliferation [16, 17]. Astrocyte and microglia purities were determined using glial fibrillary acidic protein (GFAP) and ionized calcium binding adapter molecule 1 (Iba1), respectively.

The highly aggressively proliferating immortalized (HAPI) microglia cell line purchased from Otto Biotech (Shenzhen, China) was also used in this study. This cell line was obtained initially from mixed glial cultures prepared from 3-day-old rat brain and possessed the characteristics of microglia.

2.3. Treatment of rat astrocytes with 2-CE and preparation of astrocyte-conditioned medium (ACM)

First, astrocytes were treated with 2-CE at 7.5, 15, or 30 mM for 8 h or at 30 mM from 0 to 48 h to analyze changes in target proteins. Next, the cells were treated with NAC (5, 50, or 500 μ M), FC (0.5, 1, or 2 μ M), GI (1, 5, or 10 μ M), SB202190 (1, 10, or 30 μ M), PDTC (25 μ M), or SR11302 (10 μ M) for 1 h prior to the addition of 30 mM 2-CE. These inhibitors were dissolved in dimethyl sulfoxide (DMSO) and then diluted with DMEM containing 5% FBS. The final DMSO concentration was 0.1% at most and did not affect the behavior of the cells. Moreover, the cells were transfected with siRNAs of CYP2E1, TNF- α or iNOS using Lipofectamine 3000 Transfection Reagent (Invitrogen, Carlsbad, USA) or infected with IL-1 β shRNA according to the manufacturer's instructions. Astrocytes were incubated with transfection media for 6 h, thereafter the complete media were replaced and incubated for 24 h before 2-CE exposure. The cells were harvested after 8 h treatment with 30 mM 2-CE for subsequent experiments.

For preparation of ACM, the treated astrocytes were washed with D-Hank's solution, and then incubated with fresh medium for 6 h. Subsequently, the media were collected and filtered using 0.22 μ m pore filters before addition into the cultures of primary microglia or HAPI cells. In this experiment, ACM from untreated astrocytes was used as the control. After 12 h incubation with ACM, the primary microglia and HAPI cells were collected for subsequent experiments.

2.4. Immunofluorescence

The cells were fixed with 4% paraformaldehyde for 20 min, permeabilized in 0.2% Triton for 10 min, and then blocked with 10% goat serum for 40 min at room temperature. Subsequently, astrocytes were incubated with rabbit anti-complement component 3 (C3) (1:100, ABclonal, Wuhan, China) and mouse anti-GFAP (1:250, Millipore, Billerica, USA), and microglia were incubated with rabbit anti-Iba1 (1:100, Bioss, Beijing, China), mouse anti-cluster of differentiation (CD) 86 (1:100, ABclonal), and mouse anti-arginase-1 (Arg-1) (1:100, Proteintech, Wuhan, China) at 4°C overnight. Thereafter, the secondary antibody of goat anti-Rabbit-Alexa Fluor 488 (1:100, Proteintech) and goat anti-Mouse-Alexa Fluor 597 (1:100, Proteintech) were added for a 30 min incubation at 37°C. Finally, the nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Images were obtained using a RVL-100 fluorescence microscope camera system (Echo Laboratories, USA).

2.5. Western blot

The cells were scraped and lysed in radioimmunoprecipitation assay buffer supplemented with protease and phosphatase inhibitor cocktails. Total protein concentrations were analyzed by bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Waltham, USA). Protein extracts (40 µg) were separated using 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis before being transferred to polyvinylidene difluoride membranes (Millipore). The blots were probed overnight at 4°C with the primary antibody of C3 (1:1000, ABclonal), S100A10 (1:1000, Immunoway Biotechnology, Plano, USA), IL-1β (1:1000, Abcam, Cambridge, USA), TNF-α (1:1000, Abcam), iNOS (1:500, Abcam), CYP2E1 (1:1000, Millipore), p-p38 MAPK (1:500, Cell Signaling Technology, Danvers, USA), p38 MAPK (1:1000, Cell Signaling Technology), p-p65 (1:500, Cell Signaling Technology), p-Inhibitor of κBα (p-IκBα) (1:1000, Cell Signaling Technology), p-c-Jun (1:500, Cell Signaling Technology), CD86 (1:1000, ABclonal), Arg-1 (1:1000, ABclonal), or β-actin (1:1000, ABclonal), followed by a horseradish-peroxidase-conjugated secondary antibody for 1 h. The membranes were visualized using an enhanced chemiluminescence kit. The intensity of each band was quantified via densitometry. Equal loading of protein was confirmed using β-actin.

2.6. qPCR

Total RNA in the cells was extracted using Trizol Reagent (Takara), and their concentrations were confirmed using a Nanophotometer N60 Touch (Implen, München, Germany). For the reverse transcriptase reactions, 500 ng RNA was used as a template by using the PrimeScript RT reagent kit (Takara). The expression levels of IL-1β, TNF-α, iNOS, CYP2E1, CD86, Arg-1, CD200R, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were quantified using SYBR Premix Ex Taq II (Takara) with the QuantStudio 6 Flex Real-time PCR System (Life Technologies, Carlsbad, USA). All the specific primers used for the qPCR are listed in Table 1. The running program was carried out for 30 s at 95°C followed by 40 cycles of 5 s at 95°C and 34 s at 60°C. Triplicate PCR reactions were prepared for each sample. Changes in gene expression (fold change) were calculated using the $2^{-\Delta\Delta C_t}$ method with normalization to GAPDH.

Table 1

Primer sequences for the target genes

Gene	Sequence (5'-3')		GenBank accession number
<i>IL-1β</i>	Forward	AAGAATCTATACCTGTCCTGTG	NM_031512.2
	Reverse	CATGGTGAAGTCAACTATGTC	
<i>TNF-α</i>	Forward	GGCTGTACCTTATCTACTCC	NM_012675.3
	Reverse	GGACTCCGTGATGTCTAAG	
<i>iNOS</i>	Forward	CACAGCCTCAGAGTCCTTCAT	NM_012611.3
	Reverse	ACCTTCCGCATTAGCACAGA	
<i>CYP2E1</i>	Forward	GCTACTGAACCACAAGAATGA	NM_031543.2
	Reverse	CTCCGCACATCCTTCCAT	
<i>CD86</i>	Forward	CGTCAAGACATGTGTAACCTGC	NM_020081.1
	Reverse	CTGCGCCCAAATAGTGTTTCG	
<i>Arg-1</i>	Forward	GTGGCGTTGACCTTGTCTTG	NM_017134.3
	Reverse	CCTGGTTCTGTTTCGGTTTGC	
<i>CD200R</i>	Forward	ACAGTGTTTGTACAGATGGGT	NM_023953.1
	Reverse	TCTGTCCGAGCAGTTGCTTT	
<i>GAPDH</i>	Forward	GCAAGAGAGAGGCCCTCAG	NM_017008.4
	Reverse	TGTGAGGGAGATGCTCAGTG	

2.7. Enzyme-linked immunosorbent assay (ELISA)

The levels of mature IL-1 β and TNF- α in culture media were assessed via ELISA kits (Elabscience Biotechnology, Wuhan, China) according to the manufacturer's protocol. During the co-culture of microglia with ACM, the ACM were sampled from astrocytes and detected via ELISA kits prior to being applied onto microglia.

2.8. Flow cytometry

For analysis of microglia polarization, the microglia were incubated at room temperature for 30 min with the fluorescently labeled antibodies CD86-APC (1:200, BioLegend, San Diego, USA) and CD200R-FITC (1:200, BioLegend). After incubation, the cells were washed, centrifuged and then resuspended in PBS to a final volume of 500 μ L. Flow cytometry was performed on a FACSCanto[®] apparatus (BD Biosciences, Bedford, USA). The obtained data were analyzed using FlowJo software (version 10.1, BD Biosciences).

2.9. Levels of reactive oxygen species (ROS) in the cells

The fluorescent probe of 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA, Beyotime) was used for quantifying ROS levels in the cells, and measured using a multi-functional microplate reader (BioTek Instruments, Winooski, USA) at excitation and emission wavelengths of 488 nm and 535 nm, respectively. To provide statistical data, the mean measured fluorescence intensity for the control was set to 100%. The flow cytometer was also used to fluorescently quantify the cells. The fluorescent images of ROS were visualized and acquired using a RVL-100 fluorescence microscope camera system.

2.10. Quantification of NO production

The cumulated NO production was assessed via the assay kit of Griess reaction (Elabscience Biotechnology), which detects the level nitrite (NO_2^-), a stable metabolite of NO in the cells.

2.11. Statistical analysis

Data are presented as the mean $\pm$ standard deviation (SD) and analyzed using SPSS 20.0 (IBM, Armonk, USA). Four to six independent experiments using primary cells of different origins were performed. Student's *t*-test (two-tailed) was used for comparing the differences between two groups, while one-way ANOVA followed by Student-Newman-Keuls tests were applied for determining the significant differences among multiple groups. $P < 0.05$ was defined as statistically significant.

3. Results

3.1. Alteration in astrocyte phenotype affected by treatment with 2-CE

It has been reported that C3 is a characteristic and highly upregulated marker in A1s, it is not expressed in A2s [7, 8]. In contrast, S100A10 is the marker that is upregulated in A2s [18, 19]. As shown in Figures 1a and 1b, the expression levels of C3 protein in 15 and 30 mM 2-CE treated astrocytes increased significantly compared to that in the untreated astrocytes, and those in the 30 mM 2-CE treated group were significantly higher than those in the other 2-CE treated groups. However, the expression levels of S100A10 protein increased significantly in the 7.5 mM 2-CE treated group, whereas decreased significantly in the 30 mM 2-CE treated group as compared with the control group. Moreover, the protein levels of S100A10 in 15 and 30 mM 2-CE treated cells were significantly lower than in the 7.5 mM 2-CE treated cells. These results indicate that treatment of rat astrocytes with 2-CE could dose-dependently induce A1s and suppress A2s.

On the other hand, the changes in protein levels of IL-1 β , TNF- α , and iNOS along with the doses and time of 2-CE treatment in the astrocytes were determined. The temporal profiles of IL-1 β , TNF- α , and iNOS

protein levels in the cells are illustrated in Figure 1c. The protein levels increased significantly, reached a peak and remained elevation above the control levels were at 4 h, 8 h and 12 h for IL-1 β , at 2 h, 8 h and 12 h for TNF- α , and at 4 h, 12 h and 24 h for iNOS, following the treatment with 2-CE, respectively. Accordingly, the cells were treated with 7.5, 15, and 30 mM 2-CE for 8 h. As shown in Figure 1d, the IL-1 β and TNF- α protein levels in the cells treated with 30 mM 2-CE increased significantly compared to the other groups, and the iNOS protein levels in both 15 and 30 mM 2-CE treated group were significantly higher than those in either 7.5 mM 2-CE treated group or control group. Moreover, the IL-1 β mRNA levels in all 2-CE treated groups increased significantly compared to the control group, and those in the 30 mM 2-CE treated cells were significantly higher than in the other groups (Fig. 1e). The TNF- α and iNOS mRNA levels in the cells treated with 30 mM 2-CE were significantly higher than those in either 7.5 mM 2-CE treated group or control group, and those of iNOS in 30 mM 2-CE treated group were also significantly higher than those in 15 mM 2-CE treated group (Figs. 1f and 1g). Furthermore, the levels of mature IL-1 β and TNF- α in the media from all 2-CE treated cultures increased significantly compared with those from the control culture (Figs. 1h and 1i). The concentrations of nitrite, an indicator of cumulated nitric oxide (NO) released into the media, in the media from 15 and 30 mM 2-CE treated groups increased significantly and dose dependently (Fig. 1j). All these results indicate that treatment of primary rat astrocytes with 30 mM 2-CE for 8 h could dramatically upregulate the production and release of proinflammatory mediators, such as IL-1 β , TNF- α , and iNOS due to inducing A1s.

3.2. Roles of ROS in 2-CE induced A1s

Our paper published recently reported that treatment of rat astrocytes with 2-CE could upregulate CYP2E1 expression and cause oxidative damage [20]. Accordingly, we first examined whether upregulated expression of CYP2E1 was involved in 2-CE-induced A1s. The specific siRNA was transfected into the rat astrocytes to knockdown CYP2E1 expression before treatment with 2-CE. As shown in Figures 2a and 2b, the expression levels of CYP2E1 protein and mRNA in the cells of CYP2E1 knockdown decreased significantly as compared to the control, and the negative control (NC) that was transfected with scrambled siRNA. Moreover, transfection of CYP2E1 siRNA almost completely abolished 2-CE-induced CYP2E1 overexpression (Figs. 2c and 2d), and reduced ROS production (Figs. 2e and 2f) and C3 protein upregulation, whereas enhanced the protein expression of S100A10 in astrocytes treated with 2-CE (Fig. 2g). Furthermore, we also measured the changes of C3 protein expression in the presence of NAC, a scavenger of ROS in 2-CE treated astrocytes. Pretreatment with NAC could reduce ROS production (Figs. 2h and 2i), and C3 protein expression (Figs. 2j and 2k) in the astrocytes treated with 2-CE, suggesting that ROS production might play an important role in 2-CE induced A1s.

3.3. A1s induced by 2-CE through p38 MAPK/NF- κ B and AP-1 signaling pathways

In the previous study, we have found that pretreatment of 2-CE treated rat astrocytes with NAC could attenuate the phosphorylation of p38 MAPK in these cells, suggesting that ROS production might be involved in the activation of p38 MAPK signaling pathway. Accordingly, we further explore the role of p38 MAPK signaling pathway in 2-CE induced A1s. Pretreatment of 2-CE treated astrocytes with SB202190, a specific inhibitor of the p38 MAPK could dose-dependently reverse the protein expression of C3 (Fig. 3a), and reduce the expression of IL-1 β , TNF- α , and iNOS at the transcriptional level and their secretion (Figs. 3b–h). These findings imply that the p38 MAPK signaling pathway might be involved in 2-CE induced A1s.

It has been reported that both NF- κ B and AP-1 are activated via p38 MAPK signaling pathway. Thus, we further to explore the contribution of NF- κ B or AP-1 signaling pathway to 2-CE-induced A1s by pretreatment of 2-CE treated astrocytes with PDTC, an inhibitor of NF- κ B [21] or SR11302, an inhibitor of AP-1 [22]. The alterations in expression of C3 protein, and expression and secretion of IL-1 β , TNF- α , and iNOS in either PDTC or SR11302 pretreated cells following the 2-CE treatment were similar with those achieved by the inhibitor of p38 MAPK (Figs. 3i–p). These findings suggest that treatment of rat astrocytes with 2-CE might induce A1s and upregulate the expression of proinflammatory mediators through p38 MAPK/ NF- κ B and AP-1 signaling pathways.

3.4. Effect of 2-CE induced A1s on microglia polarization

To further explore the impact of A1s on microglia, a co-culture system consisting of primary rat microglia and ACM that was from the cultures either treated or untreated with 2-CE was applied. The purity of the primary rat microglia was determined via Iba1 immunostaining (Fig. 4a). A dramatic change in the microglial morphology was observed due to the culture conditions. Microglia exposed to the ACM from 2-CE untreated astrocytes showed ramified morphology with long branches, however, the morphology of microglia exposed to the ACM from 2-CE treated astrocytes, and exposed directly to 2-CE showed reduced number of branches, branch length, and branch complexity (Fig. 4b).

It is widely used that expression of CD86 is the marker of M1 polarization [23], and Arg-1 [24, 25] or CD200R expression [26, 27] is the marker of M2 polarization in the microglial phenotype. As shown in Figure 4c, more CD86-positive microglia and fewer Arg-1-positive microglia were observed in the culture exposed to 2-CE treated ACM compared to that exposed to 2-CE untreated ACM. The expression levels of CD86 mRNA and protein increased, conversely Arg-1 decreased in the microglia exposed to the ACM from 2-CE treated cells (Fig. 4d–f).

Similar to the results reported as before, the levels of mature IL-1 β and TNF- α , and concentrations of NO in the ACM from the 2-CE treated cells were increased significantly (Figs. 4g–i). Surprisingly, although the microglia exposed directly to 30 mM 2-CE showed de-ramified morphology, the expression levels of CD86 protein decreased, but Arg-1 increased in these cells (Figs. 4b–f), suggesting the stimulated effects of ACM prepared from 2-CE-treated astrocytes on M1 polarization of microglia is not due to the 2-CE. On the

other hand, these results indicate that the primary rat microglia are not sensitive to the 2-CE effects as the primary rat astrocytes.

3.5. Factors released from the 2-CE induced A1s modulate M1 polarization of microglia

To elucidate the factors released from 2-CE-induced A1s modulate M1 polarization of microglia, the RNA interference to knock down the mRNA of IL-1 β , TNF- α , or iNOS in A1s was performed before 2-CE treatment. As shown in Figures 5a–l, the specific shRNA and siRNA effectively reduced the protein and mRNA levels of IL-1 β , TNF- α and iNOS in the cells with or without 2-CE treatment. Accordingly, the levels of mature IL-1 β , TNF- α , and concentrations of NO in the ACM prepared from shRNA infected or siRNA transfected astrocytes decreased significantly (Figs 5m–o).

It is well-known that the *in vitro* studies involving the primary microglia have been hindered by the inability to obtain high yields of purified cells due to their lack of proliferation in culture. Therefore, many *in vitro* studies were performed by using the immortalized microglia cell lines. In this study, an immortalized microglia cell line, HAPI, prepared initially from rat brain was used to further explore the factors in the ACM modulate the microglial polarization. As mentioned above, the HAPI microglial cells were co-cultured with the ACM that was prepared from the astrocytes transfected with or without the IL-1 β shRNA, TNF- α siRNA, or iNOS siRNA prior to 2-CE treatment, which were termed as NC, 2-CE, siIL-1 β , siTNF- α , and siiNOS group, respectively. The mRNA levels of CD86 decreased significantly, while the CD200R increased in the microglial cells of siIL-1 β or siTNF- α group compared to the 2-CE group (Figs. 6a–d). The percentages of CD86-positive and CD200R-positive microglial cells examined using the flow cytometry in the NC, 2-CE, siIL-1 β , siTNF- α and siNOS group were 19.0% and 2.85%, 31.7% and 2.24%, 22.7% and 2.85%, 20.8% and 2.44%, and 31.9% and 2.55%, respectively (Fig. 6g). The differences in percentage of CD86-positive cells between NC and 2-CE groups, and between either siIL-1 β or siTNF- α and 2-CE groups were significantly (Fig. 6h). Our findings from this part of study indicate that 2-CE induced A1s could stimulate M1 polarization of microglia through releasing IL-1 β and TNF- α into the ACM. Although A1s could also suppress the M2 polarization of microglia, IL-1 β and TNF- α in the ACM have no contribution to this effect (Fig. 6i). Furthermore, our results in this part of study suggest that knockdown of iNOS in A1s could not affect the microglial polarization (Figs. 6e–i).

3.6. Effects of the treatment with fluorocitrate and GIBH-130 (piperazinyI pyrimidine) on A1s, and their roles in modulation of M1 polarization of microglia

Both fluorocitrate (FC) and GIBH-130 (GI) are the inhibitors of neuroinflammation reported recently [28, 29]. Since FC is preferentially taken up by the astrocytes and can inhibit the tricarboxylic acid cycle via targeting aconitase, it is used to explore the roles of activated astrocytes under various pathological

conditions. GI is a novel anti-neuroinflammatory agent that was identified through microglia-based phenotypic screenings, however, its inhibitory effects on astrocytic activation is unclear. In the part of study, we first study the effects of either FC or GI on 2-CE induced A1s, and then further explore the alteration in M1 polarization of microglia through co-culturing microglia with the ACM prepared from the 2-CE treated astrocytes.

Our results revealed that pretreatment of rat astrocytes with either FC or GI could inhibit 2-CE-induced A1s by suppressing the expression levels of C3 protein, the specific marker of A1s (Figs. 7a–c). Moreover, pretreatment of 2-CE treated rat astrocytes with FC or GI could reduce the protein expression of IL-1 β , TNF- α , and iNOS in the cells, and their excreted levels in the culture media (Figs. 7d–k). Furthermore, pretreatment of 2-CE-exposed rat astrocytes with FC or GI could also reduce the activation of p38 MAPK/ NF- κ B and AP-1 signaling pathways by inhibiting the phosphorylation of p38 MAPK, p65, I κ B, and c-Jun proteins (Figs. 7l and 7m)

On the other hand, the primary rat microglia were examined following co-culture with the ACM prepared from 2-CE exposed astrocytes pretreated with either FC or GI. The results showed that the M1 polarization of microglia was suppressed in the FC or GI group compared to the 2-CE group, since both the proportion of CD86-positive microglia and expression levels of CD86 in the two groups decreased significantly. In contrast, the suppressed M2 polarization of microglia in the 2-CE group were relieved in the GI group as the percentages of Arg-1-positive microglia and expression levels of Arg-1 increased significantly compared to the 2-CE group. However, no significant alteration in the percentages of Arg-1-positive microglia and expression levels of Arg-1 were observed between the 2-CE and FC groups (Fig. 8a–e). Meanwhile, the levels of IL-1 β and TNF- α , and the concentrations of NO in the ACM from FC or GI pretreated cells decreased significantly compared to the 2-CE treated cells (Figs. 8f–h). These results demonstrated that the inhibition of A1s achieved by pretreatment with FC or GI could also suppress the M1 polarization of microglia through the mediators released into the ACM.

The results in this part of study provided the evidences concerning the inhibitory effects of FC and GI on 2-CE induced A1s, and indicated that IL-1 β , TNF- α , and NO in the ACM might modulate the effects of A1s on M1 polarization of microglia, which are agreement with the findings as mentioned above.

4. Discussion

Astrocytes and microglia are the important glial cells in the brain, and play the crucial roles in the biological responses to the external stimuli. Especially in the neuroinflammation, the crosstalk between the astrocytes and microglia can determine the magnitude and duration of the inflammatory responses. Increasing evidence indicates that the sustained inflammatory responses may result in the increased production of inflammatory mediators including NO, TNF- α , and IL-1 β that can directly induce the pathological changes in the brain or amplify the local inflammatory response [30–32]. Although activated microglia play the dominated roles in neuroinflammation, astrocytes can also produce and secrete the proinflammatory mediators such as NO, TNF- α , and IL-1 β , while they were activated by the external

stimuli. Therefore, it is necessary to understand the regulatory effects of activated astrocytes on activation of microglia, which are critical with respect to a wide spectrum of neurological diseases.

Based on the knowledge that astrocytes might be the first target cells and responders to 1,2-DCE in the brain, we have done a lot of *in vivo* and *in vitro* studies on the responses of astrocytes to either 1,2-DCE or 2-CE [3, 13]. Although our cumulated evidences have disclosed that the neuroinflammation is involved in 1,2-DCE induced brain edema, the molecular mechanisms underlying 1,2-DCE induced activation of astrocytes and microglia in the brain have not been well demonstrated.

In the current study, we first investigated the effects of 2-CE on the phenotype of reactive astrocytes. To our knowledge, this is the first study to reveal the role of 2-CE in A1/A2 reactive astrocytic alteration. The present results indicated that treatment of the primary rat astrocytes with the 7.5 to 30 mM 2-CE can dose-dependently stimulate A1s, and 7.5 mM 2-CE may dominantly promote the A2s. Consequently, the expression levels of IL-1 β , TNF- α , and iNOS in the 30 mM 2-CE treated astrocytes increased dramatically. However, the mRNA levels of IL-1 β in the cells, as well as the levels of IL-1 β and TNF- α in the media in 7.5 mM 2-CE treated group also increased markedly.

It is well-known that astrocytes undergo a dramatic transformation called “reactive astrocytes” and up-regulate many genes after brain injury and disease. Liddelow et al. (2017) firstly reported that two different types of reactive astrocytes, termed “A1s” and “A2s”, can be stimulated by lipopolysaccharide (LPS)-induced neuroinflammation. Many proinflammatory mediators are highly up-regulated in the A1s, which may stimulate the inflammatory response, so A1s are destructive to the recovery and repair in the brain. In contrast, many neurotrophic factors and anti-inflammatory mediators are up-regulated in the A2s, thus they are neuroprotective, and promote brain healing after injury. Accordingly, the present data suggested that treatment with 30 mM 2-CE could induce inflammation and cause damage in the brain.

Next, we investigated the ways by which 2-CE stimulates the A1s. In our previous studies, we have found that ROS produced through CYP2E1 mediated metabolism of 1,2-DCE or 2-CE can activate the p38 MAPK/ NF- κ B and AP-1 signaling pathway, and upregulate the expression of MMP-9 that may disrupt the BBB integrity, and then enhance BBB permeability and brain edema formation. Therefore, we investigated whether this signaling pathway play a role in 2-CE induced A1s.

CYP2E1 is the main enzyme that is implicated in 1,2-DCE metabolism *in vivo* [33]. Although its expression in the brain is much lower than in the liver, the enzyme is highly concentrated and inducible in the cortical astrocytes [34-37]. It has been reported that CYP2E1 can convert 1,2-DCE into 2-CE, chloroacetaldehyde and chloroacetic acid [38-41]. Because of the high oxidase activity, CYP2E1-mediated metabolism of 1,2-DCE and its intermediates can lead to the generation of ROS, as a consequence induce oxidative damage in the brain [20, 33]

The present results revealed that CYP2E1 knockdown reversed 2-CE-induced augmentation of ROS production and A1s induction, suggesting that ROS production is involved in A1 activation. Using the ROS

scavenger NAC, we further determined that ROS induce A1s. Overall, our data demonstrates for the first time that 2-CE induces A1s via ROS that was overproduced by CYP2E1-mediated metabolism of 2-CE.

Although we have reported that p38 MAPK/ NF- κ B and AP-1 signaling pathways are involved in the upregulation of MMP-9 in 2-CE treated astrocytes, whether these signaling pathways also contribute to 2-CE-induced A1s and upregulation of TNF- α , IL-1 β , and iNOS in astrocytes are unclear. This report is the first to provide strong evidence of the involvement of the p38 MAPK/ NF- κ B and AP-1 pathways in 2-CE-activated A1s. Our data indicate that as A1s are activated, p38, p65, I κ B α and c-Jun phosphorylation increases. To further explore the roles of these pathways in A1s activation, astrocytes were pretreated with the p38 inhibitor SB202190, the NF- κ B inhibitor PDTC, and the AP-1 inhibitor SR11302. The results clearly demonstrate that p38 MAPK, NF- κ B, and AP-1 regulate A1s activation and also provide evidence of these pathways in upregulating TNF- α , IL-1 β , and iNOS.

Analysis on protein and gene expression of CD86 and Arg-1, the typical marker of M1 and M2 polarization revealed marked differences in microglia treated directly with 2-CE, as well as co-cultured with the ACM prepared from 2-CE treated and untreated astrocytes. Treatment with ACM prepared from 2-CE-stimulated astrocytes significantly increased and decreased, respectively, microglial expression of CD86 and Arg-1. By contrast, the treatment of microglia with 2-CE slightly increased Arg-1 expression but decreased CD86 expression. Thus, our findings suggested that treatment of primary rat microglia with 30 mM 2-CE alone was insufficient to induce their M1 polarization, possibly because the production of ROS is less than in the astrocytes due to few expressed CYP2E1 in the microglia [34]. Furthermore, our findings demonstrated that the most possibilities of M1 polarization in microglia are activated through the mediator released by 2-CE stimulated astrocytes in the brain.

Since the aforementioned results suggested that the ACM prepared from 2-CE treated astrocytes could activate microglia, it is essential to determine the factors that play these roles in the ACM. For this aim, the gene silence target at IL-1 β , TNF- α , and iNOS was performed, and the findings indicated that the IL-1 β and TNF- α released by A1s into the ACM might contribute to microglial activation. However, the iNOS gene silence in 2-CE treated astrocytes failed to affect the microglial M1 polarization although the concentrations of NO determined in the ACM increased due to upregulated expression of iNOS. The reason is most probably due to the short half-life of NO in the culture media, where it may disappear rapidly due to oxidation and be formed nitrite [42-44]. Thus, the NO concentrations evaluated by nitrite just reflect the cumulated NO production, which is not the indicator of NO with the bioactivity. Therefore, study on co-culture of microglia with ACM can't be used to evaluate the effects of upregulated iNOS in astrocytes on microglia. For this purpose, a co-culture model by Transwell that mimics the crosstalk between cells should be used in our further study.

IL-1 β , TNF- α , and NO are the dominated inflammatory mediators that may directly induce neuronal apoptosis or amplify the local inflammatory response. Although most studies reported that they were released by microglia activation and can activate A1s, these factors may also be produced and released

by A1s. In current study, our findings for the first time demonstrated that these factors might first produced and released by 2-CE treated astrocytes, and then stimulated the microglial activation.

Both FC and GI may have the therapeutic applications in diminishing the inflammatory response of glia cells. FC, preferentially taken up by astrocytes is the reversible inhibitor of the tricarboxylic acid cycle [45]. FC may selectively induce ultrastructural alterations in astrocytes without affecting the neurons in adult male rats, thus has been widely used to inhibit astrocytes and study their roles under various pathological conditions [46, 47]. Pretreatment of mice with FC could decrease the expression of proinflammatory factors and A1s-specific transcripts in the hippocampus and cortex, and then alleviated LPS-induced depressive-like behavior [48]. It was also demonstrated that inhibiting reactive astrocytes with FC immediately before reperfusion may protect against ischemic stroke-induced memory impairment [28].

GI, the pyridazine-3-carboxamide derivative, is a novel anti-neuroinflammatory agent that has been approved by China Food and Drug Administration for clinical trials against AD. The anti-neuroinflammatory efficacy was identified via the microglia-based phenotypic screenings in AD animal models [29]. It has been reported that pretreatment with GI can selectively suppress the production of NO, TNF- α and IL-1 β through modulating MAPK signal transduction pathway in LPS-activated microglia. However, the effects of GI on astrocytic activation is unclear, and further insight into its molecular mechanisms of action is necessary.

The present results revealed that pretreatment of the primary rat astrocytes with either FC or GI could suppress A1s and activation of p38 MAPK/NF- κ B and AP-1 signaling pathways in 2-CE treated astrocytes. Moreover, FC treatment can alleviate the activation of both A1s and A2s, in contrast, GI attenuated A1s, but not A2s in 2-CE treated astrocytes. Furthermore, co-culture with the ACM prepared from either FC or GI pretreated astrocytes attenuated the microglial M1 polarization, however, GI pretreated ACM might promote microglia towards the M2 state.

Conclusions

Here the evidence from our *in vitro* studies for the first time demonstrate that treatment of rat astrocytes with 2-CE stimulate activation of A1s through ROS mediated p38 MAPK/NF- κ B and AP-1 signaling pathways, which enhance the production and secretion of IL-1 β , TNF- α , and NO; next, treatment of rat astrocytes with 2-CE can stimulate M1 polarization in microglia through IL-1 β and TNF- α released by 2-CE treated astrocytes; finally, microglial activation is less sensitive to 2-CE than astrocytes, suggesting that neuroinflammation induced by microglial activation is triggered by astrocytic activation in the brain of 1,2-DCE intoxicated mice. The understanding of the multidimensional roles of reactive astrocytes may further the development of new treatment strategies in reducing neuroinflammation and brain edema following 1,2-DCE-induced toxic encephalopathy.

Declarations

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Competing interests

No conflicts of interest, financial or otherwise, are declared by the author(s).

Availability of data and materials

Information regarding the experimental methods and data used and/or analyzed during the current study are available from the corresponding author based on reasonable requests.

Authors' contributions

JYP and WT conceived and designed the experiments. WT performed the experiments, analyzed the data, prepared the figures, and drafted the manuscript. WT and THG established the cell model. SQ, ZFH, WGY and CYH provided experimental technical support. JYP edited and revised the manuscript. All authors checked and approved the final manuscript.

Ethics approval and consent to participate

All experimental procedures were approved by the Laboratory Animal Care and Use Committee of China Medical University (IACUC: No.2020108) and were conducted according to the NIH guidelines concerning the care and use of laboratory animals.

Consent for publication

Not applicable.

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Figures

Figure 1

2-Chloroethanol (2-CE) induces A1 reactive astrocytes (A1s) and enhances the expression of interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α), and inducible nitric oxide synthase (iNOS) in A1s. (a) Protein levels of the A1-marker complement component 3 (C3) and A2-marker S100A10 in astrocytes as measured by western blotting. Top panel shows representative blots. (b) Representative immunofluorescence staining of A1-marker C3 in astrocytes (200 $\times$, scale bar = 70 μ m). (c) Western blot

was performed to delineate the time course of IL-1 β , TNF- α , and iNOS protein levels over 0-48 h. The migrating bands at 31 and 26 kDa represent precursor IL-1 β and TNF- α proteins, respectively. (d) IL-1 β , TNF- α , and iNOS protein levels in astrocytes measured by western blotting. (e–g) Quantitative polymerase chain reaction (qPCR) was used to assay the IL-1 β , TNF- α , and iNOS mRNA levels. (h, i) Enzyme-linked immunosorbent assay (ELISA) was performed to quantify IL-1 β and TNF- α levels in culture media. (j) Nitrous oxide (NO) levels in culture media as measured by the Griess reaction. (* $p < 0.05$ vs. control, # $p < 0.05$ vs. 7.5 mM 2-CE, + $p < 0.05$ vs. 15 mM 2-CE; mean $\pm$ standard deviation [SD]; one-way ANOVA followed by Student-Newman-Keuls [SNK] tests).

Figure 2

Reactive oxygen species (ROS) generated by cytochrome P4502E1 (CYP2E1)-metabolized 2-CE induce A1s. (a, b) CYP2E1 protein and mRNA levels following small interfering RNA (siRNA) transfection were assessed via western blotting and qPCR. (c, d) Effect of siRNA on 2-CE-induced CYP2E1 protein and mRNA expression. (e) Overlay map of fluorescence histograms among the different groups. ROS levels were assessed using the fluorescent probe DCFH-DA and quantified with a flow cytometer. (f) Bar graphs depicting quantitative ROS analysis. (g) C3 and S100A10 protein levels were detected via western

blotting. (h) ROS fluorescent images of astrocytes among the different groups. (i) ROS levels were quantified by measuring the mean fluorescence intensity per well. (j) Representative C3 immunostaining of astrocytes. (k) Western blot analysis for C3 in astrocytes. (* $p < 0.05$ vs. control, # $p < 0.05$ vs. 30 mM 2-CE; mean $\pm$ SD; one-way ANOVA followed by SNK tests).

Figure 3

A1s induced by 2-CE through p38 MAPK/NF- κ B and AP-1 signaling pathways. (a, b) The effects of the p38 MAPK inhibitor on C3, IL-1 β , TNF- α , and iNOS protein levels were assessed via western blotting. (c–e) The mRNA levels of IL-1 β , TNF- α , and iNOS were quantified using qPCR. (f–h) IL-1 β and TNF- α levels in media were measured using ELISA kits. NO levels in media were measured via the Griess reaction. (i, j) Effects of NF- κ B and AP-1 inhibitors on C3, IL-1 β , TNF- α , and iNOS protein levels. (k–m) IL-1 β , TNF- α , and iNOS mRNA levels. (n–p) IL-1 β , TNF- α , and NO levels in media were measured using ELISA kits or the Griess reaction. (* $p < 0.05$ vs. control, # $p < 0.05$ vs. 30 mM 2-CE, + $p < 0.05$ vs. 1 μ M SB202190, & $p < 0.05$ vs. 10 μ M SB202190; mean $\pm$ SD; one-way ANOVA followed by SNK tests).

Figure 4

Effect of 2-CE induced A1s on microglia polarization. (a) The purity of the isolated primary microglia exceeded 95% as verified by immunostaining for the microglia specific marker, ionized calcium binding adapter molecule 1 (Iba1; 100×, scale bar = 130 μm). (b) Representative phase images of primary microglia cultured with astrocyte-conditioned medium (ACM; 200×, scale bar = 70 μm). (c) Immunostaining of primary rat microglia with the M1 marker cluster of differentiation (CD) 86 and M2

marker arginase-1 (Arg-1). (d) Western blot analysis for primary rat microglial CD86 and Arg-1. (e, f) qPCR for CD86 and Arg-1 mRNA. (g–i) ELISA was performed to detect IL-1 β and TNF- α levels in ACM. The concentrations of NO in ACM were measured using the Griess reaction. (* $p < 0.05$ vs. control ACM, # $p < 0.05$ vs. 30 mM 2-CE ACM; mean $\pm$ SD; one-way ANOVA followed by SNK tests or Student's t-test).

Figure 5

IL-1 β , TNF- α , and iNOS knockdown in astrocytes are achieved via RNA interference. Successful knockdown of IL-1 β , TNF- α , and iNOS protein (a, b, c) and mRNA (d, e, f) in astrocytes as assessed via western blotting and qPCR. IL-1 β , TNF- α , and iNOS protein (g, h, i) and mRNA (j, k, l) expression levels in astrocytes following 2-CE treatment as detected by western blotting and qPCR. (m, n, o) IL-1 β , TNF- α , and NO concentrations in ACM were detected via ELISA or the Griess reaction. (* $p < 0.05$ vs. control or vs. NC, # $p < 0.05$ vs. 30 mM 2-CE; mean $\pm$ SD; one-way ANOVA followed by SNK tests).

Figure 6

2-CE induced A1s modulate M1 polarization of microglia by secreting IL-1 β and TNF- α . (a–f) Microglia phenotypes were assessed via qPCR assessment of CD86 and CD200R mRNA levels in highly aggressively proliferating immortalized (HAPI) cells. (g) Representative scatter plots depicting the gating strategy we used in the flow cytometry analysis and the percentages of CD86- and CD200R-positive subsets among the HAPI cells. (h, i) Bar graphs illustrating the quantitative analysis of CD86- and CD200R-positive microglia among the different groups. (* $p < 0.05$ vs. NC ACM, # $p < 0.05$ vs. 30 mM 2-CE ACM; mean $\pm$ SD; one-way ANOVA followed by SNK tests).

Figure 7

Effects of the treatment with fluorocitrate (FC) and GIBH-130 (piperazinyl pyrimidine, GI) on A1s. (a, b) C3 protein levels in astrocytes treated with 30 mM 2-CE and different combinations of FC or GI. (c) Double immunostaining of astrocytes with the astrocyte-marker glial fibrillary acidic protein (red) and A1-marker C3 (green) (200×, scale bar = 70 μm). (d) Effect of FC on IL-1β, TNF-α, and iNOS protein levels. (e-g) IL-1β and TNF-α levels in culture media were quantified using ELISA. NO concentrations in the culture media

were measured using the Griess reaction. (h) Effect of GI on IL-1 β , TNF- α , and iNOS protein levels. (i–k) IL-1 β and TNF- α levels in culture media were detected using ELISA, while the Griess reaction was applied to measure NO levels. (l, m) p38, p65, I κ B α , and c-Jun phosphorylation were detected using western blotting. The p38 blot depicts the same blot of p-p38, which was stripped and re-probed with an antibody against p38. (* $p < 0.05$ vs. control, # $p < 0.05$ vs. 30 mM 2-CE; mean $\pm$ SD; one-way ANOVA followed by SNK tests).

Figure 8

Roles of FC and GI in modulation of M1 polarization of microglia (a) Double immunostaining of primary microglia with the microglia-marker Iba1 (red) and the M1-marker CD86 (green) (200×, scale bar = 70 μ m). (b) Double immunostaining of primary rat microglia with Iba1 (green) and the M2-marker Arg-1 (red). (c) Western blot analysis for CD86 and Arg-1 in primary rat microglia. (d, e) qPCR for CD86 and Arg-1 mRNA. (f–h) IL-1 β , TNF- α , and NO concentrations were measured in ACM using ELISA kits or the Griess reaction. (* $p < 0.05$ vs. control ACM, # $p < 0.05$ vs. 30 mM 2-CE ACM; mean $\pm$ SD; one-way ANOVA followed by SNK tests).

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