의학석사 학위논문

Role of zinc in the induction of neurotoxic A1 astrocyte in primary mouse astrocyte culture

A1 성상세포 유도에서의 아연의 역할 규명

울 산 대 학 교 대 학 원

의 학 과

김 건 우

Role of zinc in the induction of neurotoxic A1 astrocyte in primary mouse astrocyte culture

지 도 교 수　고 재 영

이 논문을 의학석사 학위 논문으로 제출함

2021년　2월

울 산 대 학 교 대 학 원

의　학　과

김 건 우

김건우의 의학석사 학위 논문을 인준함

심사위원 고 재 영 인

심사위원 이 은 재 인

심사위원 이 승 주 인

울 산 대 학 교 대 학 원

2021년 2월

Abstract

Neurotoxic A1 astrocyte is induced by microglia-mediated neuroinflammation and plays a critical role in the neuronal damages in various neurological diseases. Therefore, factors facilitating or blocking the conversion to A1 astrocyte can be promising therapeutic targets. In this study, we demonstrate that treatment with 30 μM zinc chloride converted normal astrocytes to A1 astrocytes *in vitro* in the absence of microglia, likely by promoting inflammatory signals in the astrocytes. FluoZin-3 live-cell imaging confirmed that neuroinflammation also induces the release of intracellular zinc from metallothionein-3 (MT3) and contributes to a positive feedback loop. Neuroinflammation-mediated A1 astrocytosis was partially attenuated when intracellular zinc is depleted by co-treatment with 1 μM tetrakis (2-pyridylmethyl) ethylenediamine (TPEN), a potent zinc chelator, or genetic knockout of MT3. Collectively, our findings show that zinc plays a crucial role in the induction of A1 astrocytes by interacting with the classic neuroinflammatory pathway in a bidirectional manner.

Contents

Abstract i

Figure contents iii

Introduction 1

Methods 3

Results 6

Discussion 9

References 20

Korean abstract 25

Figure contents

Figure 1 12

Figure 2 14

Figure 3 15

Figure 4 16

Figure 5 17

Figure 6 18

Figure 7..... 19

Introduction

Astrocytes are the most common type of glial cells that tile and support the entire central nervous system (CNS). Astrocytes are involved in numerous physiologic processes, including neurotrophic support, synaptogenesis, and cerebral blood flow regulation [1, 2]. In pathological conditions such as trauma, infection, neurodegeneration, and ischemia, astrocytes undergo a profound morphological and functional change called “reactive astrocytosis”. Although reactive astrocytosis is considered the pathological hallmark of diseased CNS tissues, its definition, function, and inducing factors remain unclear [3-5].

Recent studies have revealed that reactive astrocytes have two distinct phenotypes: A1 astrocytes induced by lipopolysaccharide (LPS) treatment and A2 astrocytes induced by cerebral ischemia in mice [6, 7]. A1 astrocytes can also be induced by activated microglia, especially by pro-inflammatory cytokines such as tumor necrosis factor (TNF), interleukin 1α (IL-1α), and complement component 1q (C1q) and expressed complement 3 (C3). Since A1 astrocytes induced by these factors are highly neurotoxic and found in many neurological diseases, they were termed “neurotoxic reactive astrocytes” [8]. In the context of these findings, many studies have implicated that neurotoxic A1 astrocytes contribute to the neuronal degeneration in a variety of disorders [9-12].

Zinc (Zn^{2+}) is an essential trace metal ion that plays various roles in cell growth, synaptic transmission, and neuronal death in the CNS [13]. Cytoplasmic free zinc concentration is tightly controlled in the range of picomolar levels because it is a potent intracellular signal [14]. Metallothionein-3 (MT3), a metal-binding protein specifically expressed in the brain, has a high affinity for zinc and thus functions as a major buffer for maintaining intracellular zinc homeostasis [15]. Importantly, MT3 not only binds excessive cytotoxic zinc, but also releases free zinc in response to certain conditions and functions as an intracellular zinc reservoir [14, 16].

Recent studies have also indicated that zinc participates in neuroinflammation by modulating glial cells. For instance, zinc treatment in cultured mouse microglia triggered microglial activation

[17, 18]. Zinc administration potentiated LPS-induced pro-inflammatory cytokine production in cultured mouse microglia [19] and NFkB expression in cultured rat astrocytes [20]. Moreover, zinc chelation suppressed LPS-induced NOD-like receptor pyrin domain-containing 3 (NLRP3) inflammasome formation in co-cultured mouse neurons and astrocytes [21].

Based on these findings, we hypothesized that intracellular zinc release by MT3 contributes to the production of reactive astrocytes. Additionally, we speculated that astrocytes themselves have some capacity to be converted into a neurotoxic form without microglial support. Here, we show that zinc and MT3 play some roles in the induction of neurotoxic A1 astrocytes in primary mouse astrocyte culture.

Methods

Chemicals

Zinc chloride, TPEN, and LPS from Escherichia coli O111: B4 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Recombinant mouse TNFα (aa 80-235) was purchased from R&D Systems (410-MT; Minneapolis, MN, USA).

Astrocyte culture

Astrocytes were prepared from postnatal day 3 mice. Briefly, cerebral cortices were removed from the brains and dissociated by pipetting to produce single-cell preparations. The resulting cells were distributed in culture plates containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 7% fetal bovine serum (FBS), 7% horse serum, 100 IU/mL penicillin, and 100 mg/mL streptomycin. Primary astrocyte cultures were incubated at 37°C in a humidified 5% CO_2 atmosphere. Media were changed every 3 days with shaking to remove cells other than adherent astrocytes, and the cells were cultured until they reached confluence (14–28 days *in vitro*). All culture reagents were purchased from Invitrogen (Carlsbad, CA, USA), except for DMEM (Gibco, Gaithersburg, MD, USA) and FBS (Hyclone, Logan, UT, USA).

Western blot analysis

Cells were lysed in RIPA buffer (20 mM Tris-Cl pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1 mM EGTA, 1 μM Na_3VO_4, 2.5 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin). The lysates were centrifuged and the protein concentrations in the supernatants were determined using the bicinchoninic acid (BCA) Protein Assay Reagent (BioRad, Hercules, CA, USA). Equal amounts of protein were loaded and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% or 12% polyacrylamide gels, and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Burlington, MA, USA). The

membranes were incubated at 4°C overnight with the following primary antibodies: anti-C3 (1:1000, ab11862, Abcam, Cambridge, UK), anti-GFAP (1:5000, NB300-141, Novus, Centennial, CO, USA), anti-tubulin (1:5000, #2125, Cell Signaling, Beverly, MA, USA) and anti-TNFα (1:1000, 410-MT, R&D Systems). The membranes were then incubated with the appropriate secondary antibodies: horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:5,000, GeneTex, Irvine, CA, USA), donkey anti-goat IgG (1:2000, GeneTex), and goat anti-rat IgG (1:2000, Cell Signaling). The immunoreactive proteins were visualized by UVP Autochemi Darkroom Imaging System (Ultra-Violet Products Ltd, Cambridge, UK) and Luminata Crescendo Western HRP Substrate (Millipore).

ELISA

Cell supernatants were centrifuged and the levels of C3 and TNFα in the supernatants were measured using the appropriate ELISA kits (ab157711, ab208348, Abcam) according to the manufacturer`s instructions.

Immunocytochemistry

For immunocytochemistry, astrocytes were cultured on poly-L-Lysine-coated coverslips using the same culture methods described in “Astrocyte culture”. Primary cortical cell cultures were fixed with 4% paraformaldehyde for 30 minutes and permeabilized for 10 minutes with 0.5% saponin containing 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). Fixed and permeabilized cells were blocked with 2% BSA, and incubated overnight at 4°C with the following primary antibodies: anti-C3 (1:1000, ab11862, Abcam) and anti-GFAP (1:4000, MAB360, Millipore). The cells were then incubated with Alexa Fluor555-goat anti-rat IgG (1:1000, Invitrogen) or Alexa Fluor488-donkey anti-mouse IgG (1:1000, Invitrogen) for 2 hours, and Hoechst 33342 (1:5000, Invitrogen) for 10 minutes at room temperature. Images were obtained by a confocal microscopic imaging system (Carl Zeiss, LSM 780, Zen software, Oberkochen, Germany).

Live-cell zinc fluorescence assay

Astrocytes cultured on poly-L-Lysine-coated coverslips were stained with the zinc-specific fluorescent dye Fluozin-3-AM (Molecular Probes, Eugene, OR, USA). Equimolar concentrations of FluoZin-3 and pluronic acid were added to cells at a final concentration of 2.5 μM and incubated for 30 minutes at 37°C in a humidified CO_2 incubator. The cells were then washed with minimum essential medium (MEM, Gibco) and assessed with a confocal microscopic imaging system (Carl Zeiss, LSM 780, Zen software).

Statistical analysis

All results are presented as mean ± standard error of the mean. Two-tailed Student's *t*-test was used to evaluate the significance of the differences between groups. Differences with P-values <0.05 were considered statistically significant. Statistical analyses were conducted using IBM SPSS Statistics for Windows, version 24.0 (IBM Corp., Armonk, NY, USA). Graphical presentations were created by GraphPad Prism, version 9 (GraphPad Software, La Jolla, CA, USA).

Results

Zinc induces the conversion of primary astrocytes to neurotoxic A1 astrocyte

We first confirmed the zinc-mediated induction of A1 astrocytes by treating primary mouse astrocyte cultures with 30 μM zinc chloride. After six hours, the levels of C3 (neurotoxic A1 astrocyte marker) and glial fibrillary acidic protein (GFAP; global astrocyte marker) [22, 23] in Western blot analysis (Figure 1A-C) were significantly higher in zinc-treated cells compared with control cells, suggesting that zinc induces reactive astrocytosis and the production of A1 astrocytes. Importantly, pre-treatment with 1 μM tetrakis (2-pyridylmethyl) ethylenediamine (TPEN), a potent cell membrane-permeable zinc chelator [24] at 1 hour before zinc treatment effectively blocked the zinc-induced increases in C3; in contrast, the increases in GFAP was not significantly affected by pre-treatment with TPEN. Extracellular C3 levels in the cell supernatants were also measured by ELISA under a similar experimental condition except for incubation time (24 hours) (Figure 1D), which showed similar results to the Western blot analysis in terms of zinc-mediated increases in C3 levels and the effect of TPEN pre-treatment.

We then immunostained the astrocytes treated with 30 μM for 24 hours and visualized them using confocal microscopy for C3 and GFAP (Figure 2A). C3-positive cells are considered as reactive A1 astrocytes because non-reactive astrocytes hardly express C3 [7]. Because our study was based on primary astrocyte culture, all cells were GFAP-positive. In the quantification analysis (Figure 2B-D), the fluorescence intensity of C3 and the proportion of A1 astrocytes were significantly higher in the zinc-treated group, which was effectively reversed by TPEN pre-treatment. Our results thus far demonstrated that zinc induces A1 astrocytes in primary astrocyte culture.

Zinc induces the production of pro-inflammatory cytokine TNFα

TNFα released by activated microglia is one of the most important cytokines for A1 astrocyte induction [8]. Therefore, to investigate the mechanism of zinc-mediated induction of A1 astrocytes,

we measured the level of TNFα in astrocytes treated with 30 μM zinc using Western blot (Fig. 3A, B) and ELISA (Fig. 3C). In Western blot, the addition of TPEN significantly reduced the level of TNFα compared with zinc-treated astrocytes. In ELISA, treatment with zinc significantly increased the level of TNFα compared with control. Therefore, our results show that zinc likely converts normal astrocyte into neurotoxic A1 astrocyte by increasing the level of secreted pro-inflammatory cytokines such as TNFα. This is particularly interesting because while TNFα is usually released by activated microglia, our culture system solely consists of astrocytes; this suggests that zinc may induce the release of TNFα from astrocytes to induce neurotoxic A1 astrocytes without microglial support.

TNFα promotes intracellular zinc release by metallothionein-3

A recent study showed that neuroinflammation induces zinc dyshomeostasis in neurons and astrocytes [21], which is important because alteration of intracellular zinc level and the accompanying neuroinflammation is evident in various pathological conditions of the CNS [25]. Considering this, we took a backward approach and hypothesized that the neuroinflammatory signals induce excessive accumulation of intracellular zinc in astrocytes, which in turn induces the conversion to neurotoxic A1 astrocytes.

Primary astrocyte cultures were exposed to 20 ng/ml TNFα for 30 minutes and stained with FluoZin-3-AM, a zinc-sensitive fluorescent dye. Live-cell confocal images were obtained every 10 minutes until 50 minutes (Figure 4A), which showed a substantial accumulation of intracellular free zinc in response to TNFα. To determine the source of intracellular zinc release, we performed the same experiment in primary astrocyte cultures obtained from mice with genetic deletion of MT3, a major intracellular zinc reservoir [14]. The zinc-releasing pattern of MT3 knockout (KO) astrocytes was different from that of wild-type (WT) mice (Figure 4B-C), showing a lesser amount of released zinc and a slower increasing slope. Thus, our results suggest that neuroinflammatory signal such as TNFα promotes intracellular zinc accumulation in astrocytes, and that MT3 acts as a major source of

zinc in such condition.

Zinc chelation and MT3 KO partially attenuates TNFα-related induction of neurotoxic A1 astrocytes

To further explore the role of intracellular zinc released by neuroinflammatory signals, we assessed whether zinc chelation or intracellular zinc depletion by MT3 KO suppresses the TNFα-induced induction of A1 astrocytes. MT3 WT and KO primary astrocyte cultures were treated with 20 ng/ml TNFα with or without 1 μM TPEN for 6 or 24 hours and underwent Western blot analysis and ELISA, respectively (Figure 5). In both analyses, C3 was significantly increased by TNFα, and this change was attenuated by co-treatment with TPEN. Notably, the effect of MT3 KO was relatively small compared with that of TPEN. The changes in the GFAP levels seemed to be similar to C3, albeit without statistical significance.

Immunocytochemistry showed profound induction of A1 astrocytes by TNFα treatment (Figure 6A). Interestingly, MT3 KO astrocytes showed less C3 fluorescence intensity compared with MT3 WT astrocytes at both baseline and after TNFα treatment (Figure 6B). GFAP fluorescence intensity and the proportion of A1 astrocytes were not significantly different between MT3 WT and KO astrocytes (Figure 6C-D). TPEN co-treatment did not show significant effects in all quantification analyses.

Collectively, our results show that zinc chelation by TPEN and intracellular zinc depletion by MT3 KO partially block the TNFα-induced A1 astrocyte conversion in primary astrocyte culture. Therefore, zinc seems to play a role in the neuroinflammation-mediated induction of neurotoxic A1 astrocytes.

Discussion

Neurotoxic A1 astrocytes are an emerging key concept in various neuropathological conditions, and are induced by a combination of TNF, IL-1α, and C1q produced by activated microglia. Importantly, the A1 astrocytes induce intense neurotoxicity and do not confer many of the normal astrocytic functions. A1 astrocytes strongly upregulate many classical complement cascade genes including C3, which is exclusively expressed in A1 phenotypes and thus considered as a specific marker of A1 astrocytes. Conversely, A2 astrocytes, another phenotype of reactive astrocytes, are considered to be neuroprotective since they upregulate many neurotrophic factors [6-8]. Recent studies have investigated the mechanism of conversion to A1 astrocytes because the reversal or blockage of A1 astrocytosis can be a novel, effective therapeutic target in preventing neuronal damage [9, 26, 27].

In this study, we show that zinc treatment converts normal astrocytes to neurotoxic A1 astrocytes in primary culture without microglial involvement. The exact mechanism of this phenomenon is uncertain, but a possible explanation based on our results is that intracellular zinc accumulation induces the production of inflammatory cytokines such as TNFα from astrocytes. This finding is consistent with the latest evidence that the astrocyte itself has some inflammation-inducing potential, which is mediated by zinc [21]. Also, previous reports suggested that zinc produces inflammatory cytokines by activating the NFκB signaling pathway in human lymphoma cells [28, 29]. Considering that NFκB is a pivotal mediator of inflammatory responses [30] and is considered to play a role in neuroinflammation-mediated A1 astrocyte conversion [27], zinc may induce a pro-inflammatory state in normal astrocytes via the NFκB signaling pathway, which finally converts them to neurotoxic A1 phenotype.

Furthermore, our study suggests that the relationship between zinc and neuroinflammation is not unidirectional. The action of TNFα, one of the most powerful inducers of A1 astrocytes, was partially blocked by intracellular zinc chelation and the genetic deletion of intracellular zinc reservoir, MT3. In

addition, MT3-null astrocytes showed less "A1-like features" compared with control astrocytes. These results suggest that A1 astrocyte conversion by neuroinflammation works in a zinc-dependent manner, at least in part. Thus, zinc contributes to A1 astrocytosis in two ways that form a positive feedback loop—zinc promotes inflammatory signals in astrocytes, and neuroinflammation also facilitates the release of zinc release from MT3; yet, it is still unclear whether zinc alone, separate from the inflammatory process, can induce A1 astrocytes (Figure 7).

An excessive amount of zinc induces neurotoxicity in traumatic brain injury [31, 32] and cerebral ischemia models [33]. Several mechanisms of zinc-mediated neurotoxicity have been reported, including oxidative stress [34-36], glycolysis inhibition [37], and the promotion of apoptosis [38]. Our current study results add to the mechanisms of zinc-mediated neurotoxicity—"zinc-mediated A1 astrocyte induction". This mechanism is unique because most previous explanations of the neurotoxicity of zinc were focused on pathologic zinc accumulation in the neurons. However, in pathologic conditions such as cerebral ischemia, a large amount of free zinc is released extracellularly [39], which affects not only neurons but also glial cells. Whereas astrocytes were largely regarded as buffers or protectors in the conditions of zinc dyshomeostasis, our study implies that astrocytes can act as attackers under certain conditions of excessive zinc.

There are some limitations to our study. First of all, we only analyzed the A1 phenotype in this study by using C3 as the A1 marker, and GFAP as a pan-reactive marker. Notably, these two reactive astrocyte markers were not always correlated, which suggests the presence of other subtypes of astrocytic activation such as A2 phenotype. Further studies analyzing A2 markers are needed, including tumor growth factor β or S100A10 [27]. Secondly, we only analyzed TNFα as the pro-inflammatory cytokine in the induction of the A1 astrocytes. Classically, a combination of TNF, IL-1α, and C1q could induce the A1 astrocytes and each cytokine alone showed a weaker potency of A1 astrocyte induction [8]. Finally, our results are confined to *in vitro* model. Although this experimental setting conferred the advantage of excluding the effects of other cells such as microglia, further

investigation using *in vivo* models is required to confirm the results.

In conclusion, we demonstrate that zinc contributes to neurotoxic A1 astrocyte conversion *in vitro*. Zinc induces A1 astrocyte by triggering inflammation in astrocytes, but also plays some roles in the lower steps of classic neuroinflammation-mediated A1 astrocyte induction. Chelating excess zinc in astrocytes can be a novel therapeutic target for neuroprotection.

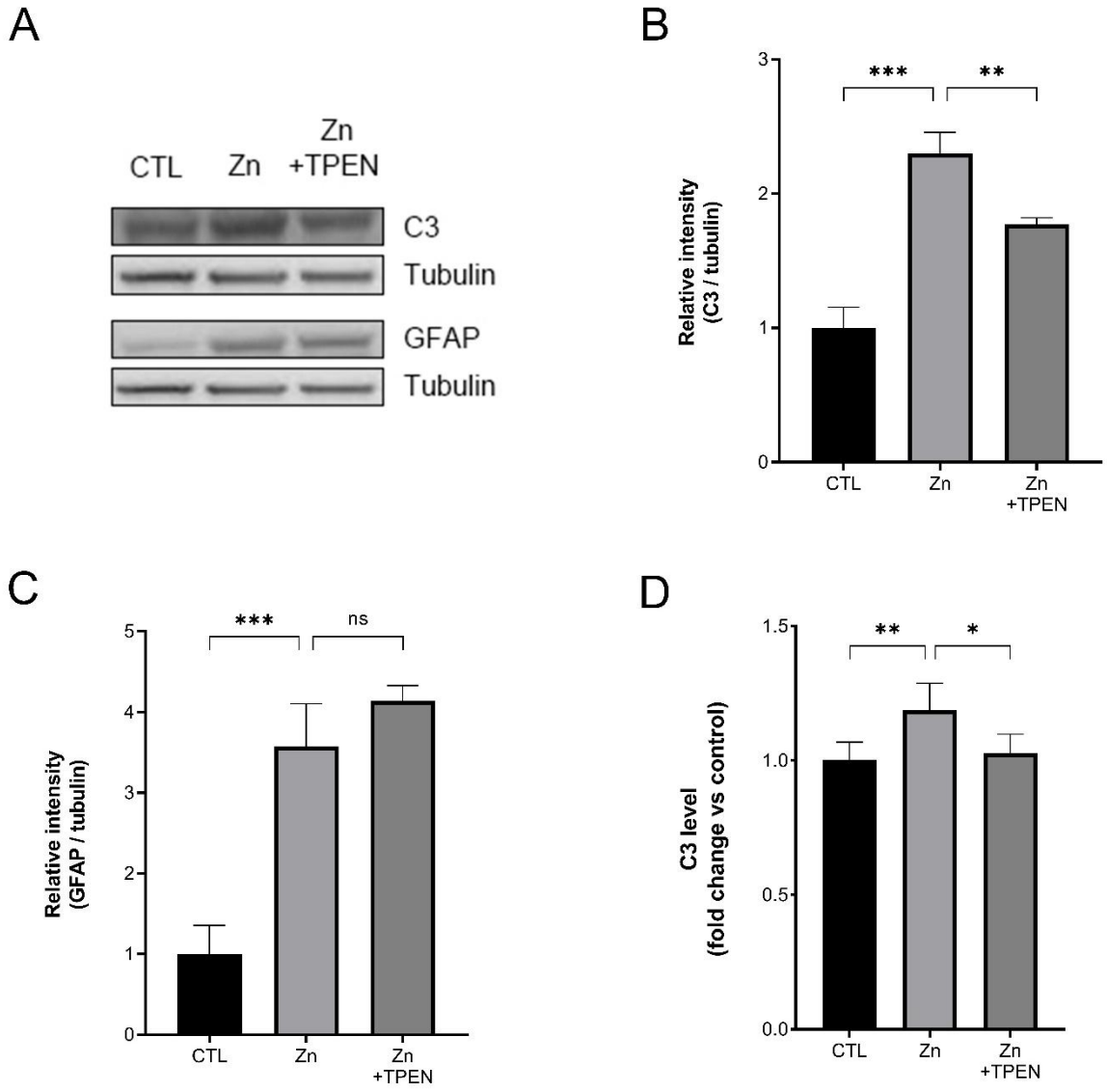

Figure 1. Increases in reactive astrocytic markers by zinc treatment

(A) Western blot analysis of zinc-treated primary astrocyte culture. Expression levels of neurotoxic A1 astrocyte marker C3 and reactive astrogliosis marker GFAP in response to zinc treatment with or without TPEN are shown. (B, C) Quantification of the relative intensities of C3 (CTL, n=5; Zn, n=5; Zn+TPEN, n=3) and GFAP (CTL, n=4; Zn, n=4; Zn+TPEN, n=3; **P<0.01, ***P<0.001). (D) ELISA for C3 in the supernatants of zinc-treated astrocytes (CTL, n=7; Zn, n=6; Zn+TPEN, n=4; *P<0.05, **P<0.01).

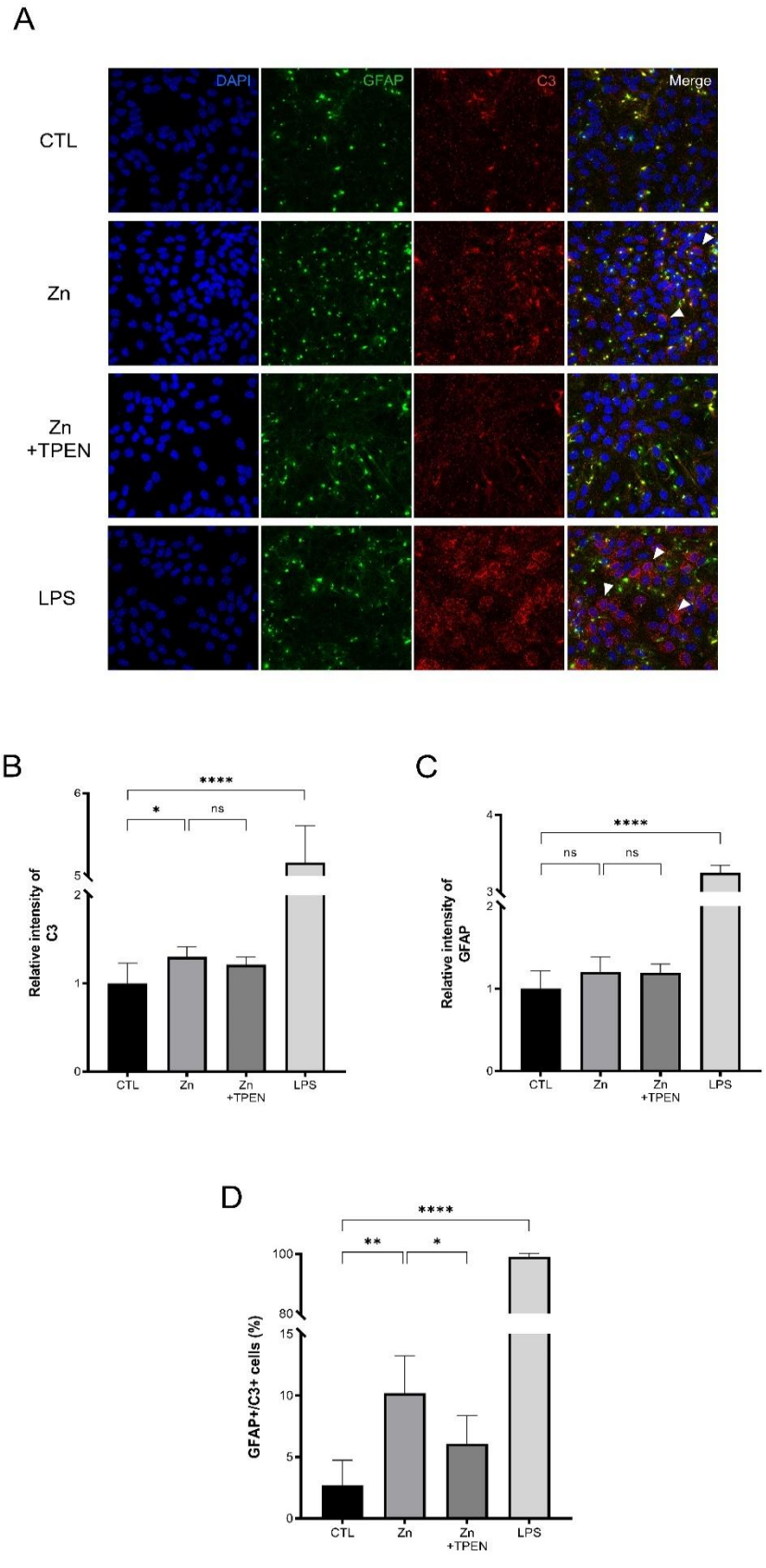
A
DAPI
GFAP
C3
Merge
CTL
Zn
Zn +TPEN
LPS
B
Relative intensity of C3
****
*
ns
CTL
Zn
Zn +TPEN
LPS
C
Relative intensity of GFAP
****
ns
ns
CTL
Zn
Zn +TPEN
LPS
D
GFAP+/C3+ cells (%)
****
**
*
CTL
Zn
Zn +TPEN
LPS

Figure 2. Zinc induces the conversion to neurotoxic A1 astrocytes in primary mouse astrocyte culture

(A) Confocal microscopic imaging of zinc-treated primary astrocyte culture (n=3 per condition). 10 μg/ml LPS was used as a positive control. DAPI staining (nuclei) is shown in blue. Colocalization of neurotoxic A1 phenotypes (white arrowheads) with GFAP (green) and C3 (red) are shown. (B, C) Relative fluorescence intensities using region-of-interest analysis (*P<0.05, ****P<0.0001). (D) Proportion of $GFAP^+/C3^+$ cells (A1 astrocytes) among total cells (*P<0.05, **P<0.01, ****P<0.0001).

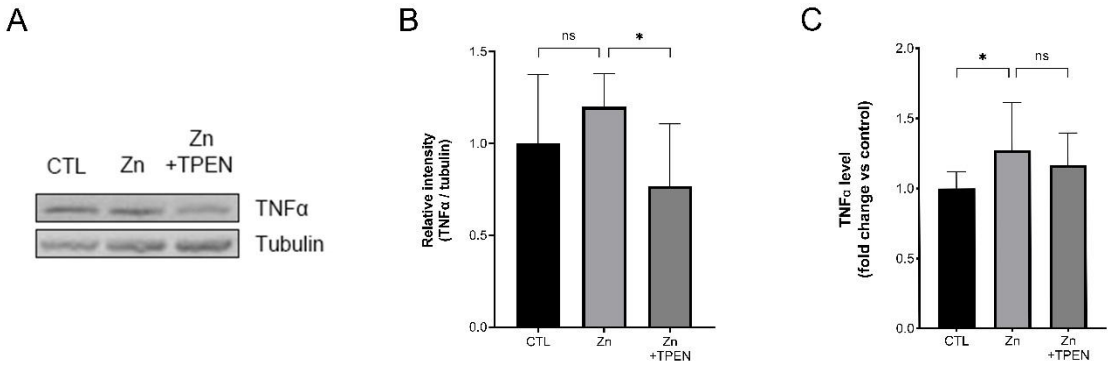

Figure 3. Zinc-mediated TNFα induction in astrocytes

(A) Western blot analysis showing TNFα elevation by zinc treatment. (B) Quantification of the relative intensities of TNFα and tubulin (CTL, n=7; Zn, n=7; Zn+TPEN, n=2). (C) ELISA for TNFα after zinc treatment (n=6 per condition, *P<0.05).

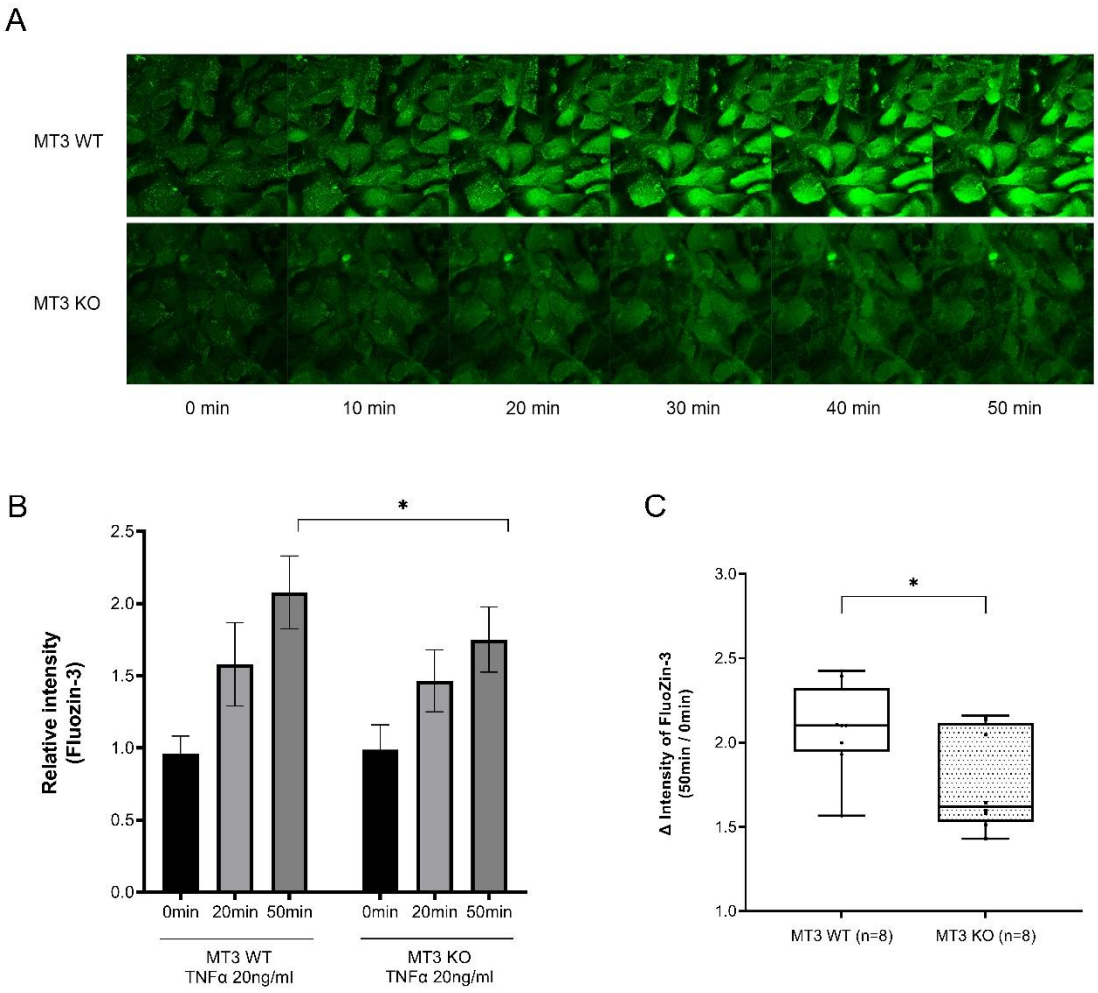

Figure 4. TNFα induces the accumulation of intracellular zinc from metallothionein-3

(A) FluoZin-3 live-cell confocal images of MT3 WT and KO astrocytes. (B) Quantification of FluoZin-3 fluorescence by region-of-interest analysis at 0 min, 20 min, and 50min (n=8 per condition, *P<0.05). (C) Ratio of fluorescence in 50 min to 0 min in MT3 WT and MT3 KO astrocytes (*P<0.05).

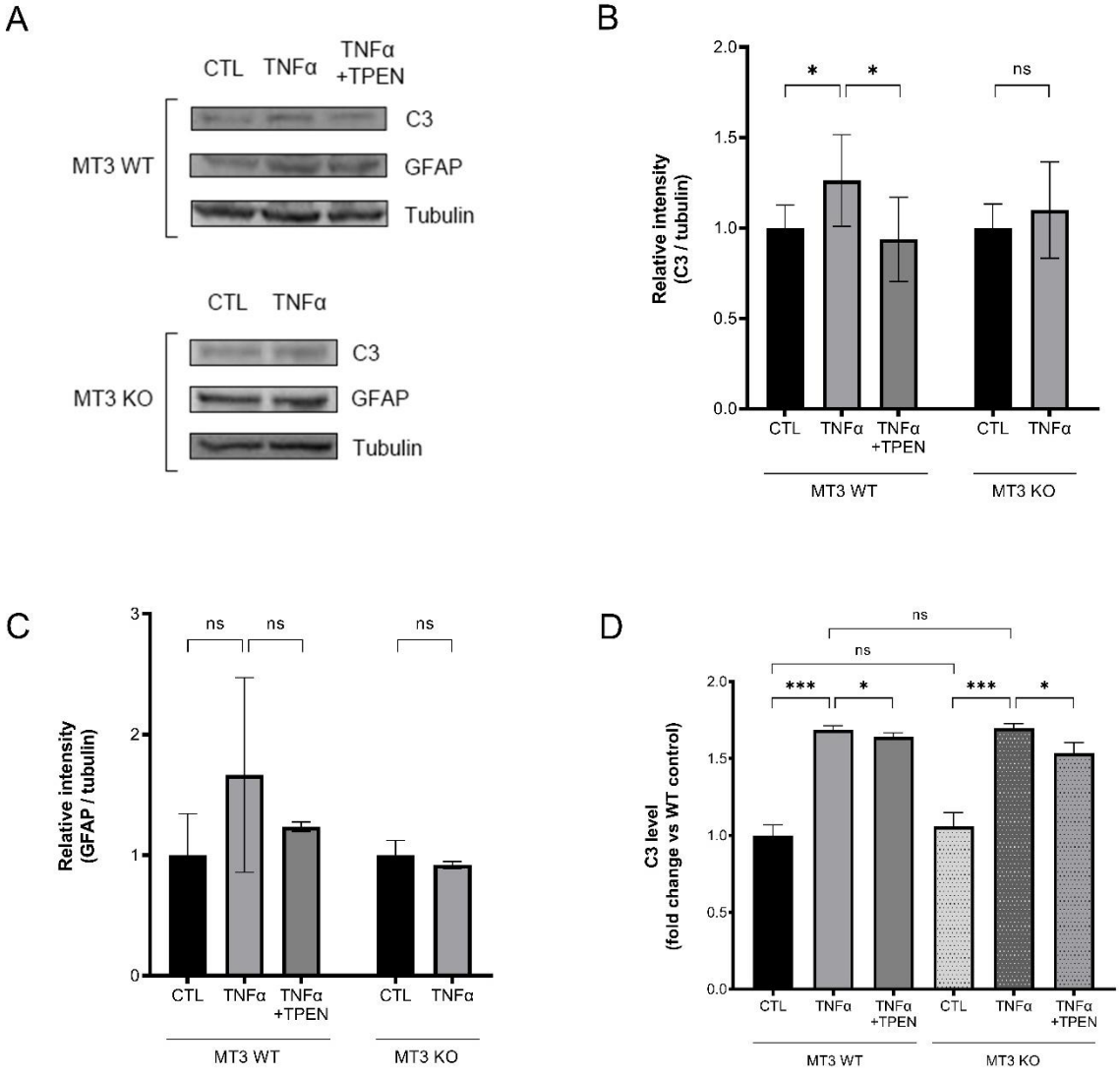

Figure 5. Blocking of TNFα-mediated A1 astrocyte induction by TPEN and MT3 knockout

(A) Western blot analysis demonstrating C3 and GFAP induction by TNFα, which is attenuated by TPEN co-treatment and MT3 knockout. (B) Quantification analysis of C3 (WT CTL, n=7; WT TNFα, n=7; WT TNFα+TPEN, n=5; KO CTL, n=2; KO TNFα, n=2; *P<0.05). (C) Quantification analysis of GFAP (WT CTL, n=4; WT TNFα, n=4; WT TNFα+TPEN, n=3; KO CTL, n=2; KO TNFα, n=2). (D) ELISA of C3 levels in MT3 WT and MT3 KO astrocytes.

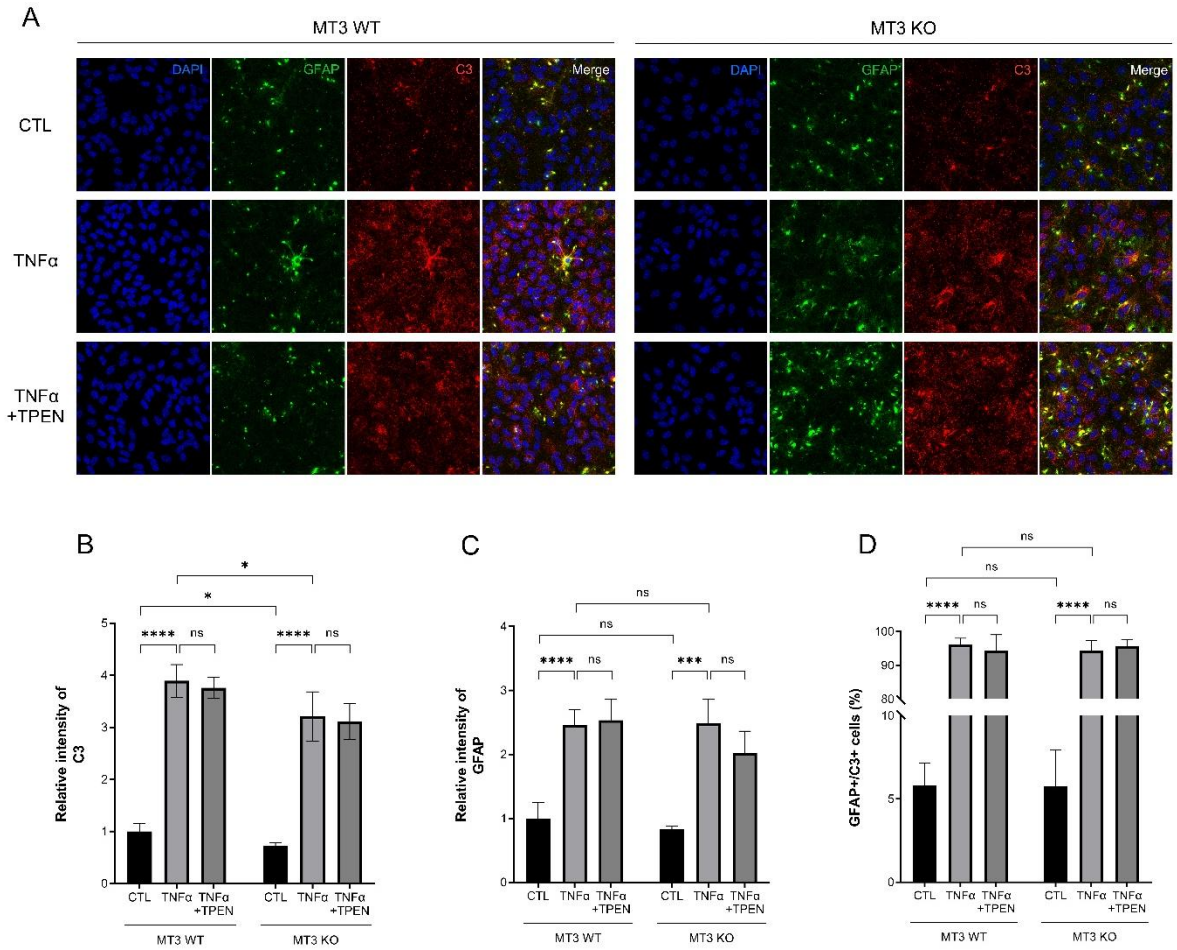

Figure 6. Immunocytochemistry of TNFα-mediated A1 astrocyte induction

(A) Confocal microscopic imaging of TNFα-treated primary astrocyte cultures (n=3 per condition). (B, C) Region-of-interest analysis of C3 and GFAP (*P<0.05, ***P<0.001, ****P<0.0001). (D) Proportion of neurotoxic A1 astrocytes in each experimental group (****P<0.0001).

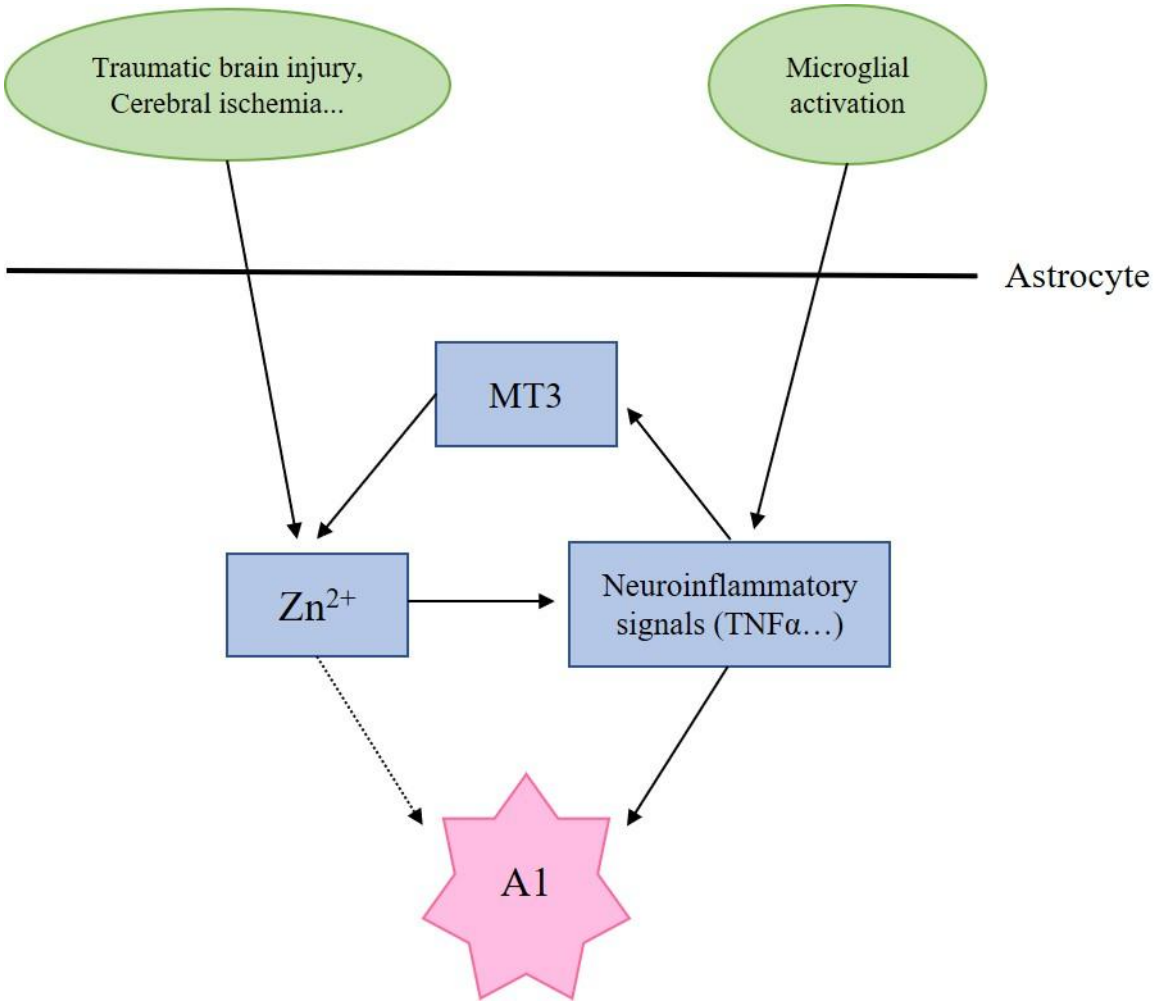

Figure 7. Schematic diagram of zinc-mediated induction of A1 astrocytes

In astrocytes, excessive zinc accumulation induces the conversion to A1 astrocytes. Zinc and neuroinflammation form a positive feedback loop. The main intracellular source of zinc is MT3. Astrocytes could be converted into A1 astrocytes by zinc dyshomeostasis even in the absence of microglial involvement. Whether zinc can directly induce the conversion to A1 astrocytes is unclear (dotted line).

References

1. Sofroniew MV and Vinters HV, Astrocytes: biology and pathology. Acta Neuropathol, 2010. 119(1): p. 7-35.
2. Liddelow S and Barres B, SnapShot: Astrocytes in Health and Disease. Cell, 2015. 162(5): p. 1170-1170.e1171.
3. Pekny M and Pekna M, Astrocyte reactivity and reactive astrogliosis: costs and benefits. Physiol Rev, 2014. 94(4): p. 1077-1098.
4. Sofroniew MV, Molecular dissection of reactive astrogliosis and glial scar formation. Trends Neurosci, 2009. 32(12): p. 638-647.
5. Wilhelmsson U, Bushong EA, Price DL, Smarr BL, Phung V, Terada M, et al., Redefining the concept of reactive astrocytes as cells that remain within their unique domains upon reaction to injury. Proc Natl Acad Sci U S A, 2006. 103(46): p. 17513-17518.
6. Zamanian JL, Xu L, Foo LC, Nouri N, Zhou L, Giffard RG, et al., Genomic analysis of reactive astrogliosis. J Neurosci, 2012. 32(18): p. 6391-6410.
7. Liddelow SA and Barres BA, Reactive Astrocytes: Production, Function, and Therapeutic Potential. Immunity, 2017. 46(6): p. 957-967.
8. Liddelow SA, Guttenplan KA, Clarke LE, Bennett FC, Bohlen CJ, Schirmer L, et al., Neurotoxic reactive astrocytes are induced by activated microglia. Nature, 2017. 541(7638): p. 481-487.
9. Yun SP, Kam TI, Panicker N, Kim S, Oh Y, Park JS, et al., Block of A1 astrocyte conversion by microglia is neuroprotective in models of Parkinson's disease. Nat Med, 2018. 24(7): p. 931-938.
10. Peng AYT, Agrawal I, Ho WY, Yen YC, Pinter AJ, Liu J, et al., Loss of TDP-43 in astrocytes leads to motor deficits by triggering A1-like reactive phenotype and triglial dysfunction. Proc Natl Acad Sci U S A, 2020. 117(46): p. 29101-29112.

11. Gibson EM, Nagaraja S, Ocampo A, Tam LT, Wood LS, Pallegar PN, et al., Methotrexate Chemotherapy Induces Persistent Tri-glial Dysregulation that Underlies Chemotherapy-Related Cognitive Impairment. Cell, 2019. 176(1-2): p. 43-55.e13.
12. Grimaldi A, Pediconi N, Oieni F, Pizzarelli R, Rosito M, Giubettini M, et al., Neuroinflammatory Processes, A1 Astrocyte Activation and Protein Aggregation in the Retina of Alzheimer's Disease Patients, Possible Biomarkers for Early Diagnosis. Front Neurosci, 2019. 13: p. 925.
13. Choi S, Hong DK, Choi BY, and Suh SW, Zinc in the Brain: Friend or Foe? Int J Mol Sci, 2020. 21(23).
14. Maret W, Molecular aspects of human cellular zinc homeostasis: redox control of zinc potentials and zinc signals. Biometals, 2009. 22(1): p. 149-157.
15. Bousleiman J, Pinsky A, Ki S, Su A, Morozova I, Kalachikov S, et al., Function of Metallothionein-3 in Neuronal Cells: Do Metal Ions Alter Expression Levels of MT3? Int J Mol Sci, 2017. 18(6).
16. Koh JY and Lee SJ, Metallothionein-3 as a multifunctional player in the control of cellular processes and diseases. Mol Brain, 2020. 13(1): p. 116.
17. Ueba Y, Aratake T, Onodera KI, Higashi Y, Hamada T, Shimizu T, et al., Attenuation of zinc-enhanced inflammatory M1 phenotype of microglia by peridinin protects against short-term spatial-memory impairment following cerebral ischemia in mice. Biochem Biophys Res Commun, 2018. 507(1-4): p. 476-483.
18. Kauppinen TM, Higashi Y, Suh SW, Escartin C, Nagasawa K, and Swanson RA, Zinc triggers microglial activation. J Neurosci, 2008. 28(22): p. 5827-5835.
19. Higashi Y, Aratake T, Shimizu S, Shimizu T, Nakamura K, Tsuda M, et al., Influence of extracellular zinc on M1 microglial activation. Sci Rep, 2017. 7: p. 43778.
20. Moriyama M, Fujitsuka S, Kawabe K, Takano K, and Nakamura Y, Zinc Potentiates

Lipopolysaccharide-induced Nitric Oxide Production in Cultured Primary Rat Astrocytes. Neurochem Res, 2018. 43(2): p. 363-374.

21. Park HS, Yoo MH, and Koh JY, Role of zinc dyshomeostasis in inflammasome formation in cultured cortical cells following lipopolysaccharide or oxygen-glucose deprivation/reperfusion exposure. Neurobiol Dis, 2020. 137: p. 104771.

22. Li S, Uno Y, Rudolph U, Cobb J, Liu J, Anderson T, et al., Astrocytes in primary cultures express serine racemase, synthesize d-serine and acquire A1 reactive astrocyte features. Biochem Pharmacol, 2018. 151: p. 245-251.

23. Clarke LE, Liddelow SA, Chakraborty C, Münch AE, Heiman M, and Barres BA, Normal aging induces A1-like astrocyte reactivity. Proc Natl Acad Sci U S A, 2018. 115(8): p. E1896-e1905.

24. Huang Z, Zhang XA, Bosch M, Smith SJ, and Lippard SJ, Tris(2-pyridylmethyl)amine (TPA) as a membrane-permeable chelator for interception of biological mobile zinc. Metallomics, 2013. 5(6): p. 648-655.

25. Sensi SL, Paoletti P, Koh JY, Aizenman E, Bush AI, and Hershfinkel M, The neurophysiology and pathology of brain zinc. J Neurosci, 2011. 31(45): p. 16076-16085.

26. Wang J, Hou Y, Zhang L, Liu M, Zhao J, Zhang Z, et al., Estrogen Attenuates Traumatic Brain Injury by Inhibiting the Activation of Microglia and Astrocyte-Mediated Neuroinflammatory Responses. Mol Neurobiol, 2020.

27. Xu X, Zhang A, Zhu Y, He W, Di W, Fang Y, et al., MFG-E8 reverses microglial-induced neurotoxic astrocyte (A1) via NF-κB and PI3K-Akt pathways. J Cell Physiol, 2018. 234(1): p. 904-914.

28. Bao B, Prasad A, Beck FW, Suneja A, and Sarkar F, Toxic effect of zinc on NF-kappaB, IL-2, IL-2 receptor alpha, and TNF-alpha in HUT-78 (Th(0)) cells. Toxicol Lett, 2006. 166(3): p. 222-228.

29. Bao B, Prasad AS, Beck FW, and Sarkar FH, Zinc up-regulates NF-kappaB activation via

phosphorylation of IkappaB in HUT-78 (Th0) cells. FEBS Lett, 2007. 581(23): p. 4507-4511.

30. Oeckinghaus A and Ghosh S, The NF-kappaB family of transcription factors and its regulation. Cold Spring Harb Perspect Biol, 2009. 1(4): p. a000034.

31. Suh SW, Chen JW, Motamedi M, Bell B, Listiak K, Pons NF, et al., Evidence that synaptically-released zinc contributes to neuronal injury after traumatic brain injury. Brain Res, 2000. 852(2): p. 268-273.

32. Hellmich HL, Eidson KA, Capra BA, Garcia JM, Boone DR, Hawkins BE, et al., Injured Fluoro-Jade-positive hippocampal neurons contain high levels of zinc after traumatic brain injury. Brain Res, 2007. 1127(1): p. 119-126.

33. Kitamura Y, Iida Y, Abe J, Mifune M, Kasuya F, Ohta M, et al., Release of vesicular Zn2+ in a rat transient middle cerebral artery occlusion model. Brain Res Bull, 2006. 69(6): p. 622-625.

34. Noh KM and Koh JY, Induction and activation by zinc of NADPH oxidase in cultured cortical neurons and astrocytes. J Neurosci, 2000. 20(23): p. Rc111.

35. Kim YH, Eom JW, and Koh JY, Mechanism of Zinc Excitotoxicity: A Focus on AMPK. Front Neurosci, 2020. 14: p. 577958.

36. Kim YH and Koh JY, The role of NADPH oxidase and neuronal nitric oxide synthase in zinc-induced poly(ADP-ribose) polymerase activation and cell death in cortical culture. Exp Neurol, 2002. 177(2): p. 407-418.

37. Sheline CT, Behrens MM, and Choi DW, Zinc-induced cortical neuronal death: contribution of energy failure attributable to loss of NAD(+) and inhibition of glycolysis. J Neurosci, 2000. 20(9): p. 3139-3146.

38. Eom JW, Lee JM, Koh JY, and Kim YH, AMP-activated protein kinase contributes to zinc-induced neuronal death via activation by LKB1 and induction of Bim in mouse cortical cultures. Mol Brain, 2016. 9: p. 14.

39. Koh JY, Suh SW, Gwag BJ, He YY, Hsu CY, and Choi DW, The role of zinc in selective

neuronal death after transient global cerebral ischemia. Science, 1996. 272(5264): p. 1013-1016.

Korean abstract

성상세포는 염증성 또는 허혈성 자극에 의해 반응성 성상세포로 활성화되어 그 기능과 역할이 바뀔 수 있음이 알려져 있다. 특히 최근에는 미세아교세포에 의해 TNFα, IL-1α, C1q 등의 염증성 사이토카인 자극을 받아 활성화된 반응성 성상세포인 A1 성상세포가 신경독성을 나타내 신경세포의 사멸에 직접적으로 관여함이 밝혀졌다. 그러나 미세아교세포에서 전달하는 염증성 자극만이 A1 성상세포를 유도할 수 있는 것인지, 다른 기전도 관여하는 것인지에 대해서는 아직 충분히 연구되어 있지 않다. 이에 본 연구에서는 A1 성상세포의 발현에 아연이 어떠한 역할을 하는지 조사하였다.

배양된 생쥐의 성상세포에 아연을 처리하였을 때 A1 성상세포의 표지자인 C3 단백질 발현이 증가하였고, 성상세포들이 반응성 성상세포들로 활성화됨을 시사하는 GFAP 증가 소견도 관찰되었다. 또한, 아연에 의해 성상세포에서 TNFα 발현이 증가함이 관찰되어, 아연이 미세아교세포 없이도 성상세포의 염증반응을 유도하여 A1 성상세포로 전환시킬 수 있음을 시사하였다. 반대로, 성상세포에 TNFα 를 처리하였을 때 성상세포의 세포 내 아연 농도가 증가함이 관찰되었고, 이러한 효과는 세포 내 아연 농도 조절에 관여하는 단백질인 metalloprotein-3 (MT3)를 knockout 시켰을 때 감소하였다. 마지막으로 TNFα 에 의한 A1 성상세포 유도가 MT3 knockout 또는 zinc chelation 에 의해 부분적으로 억제됨을 확인하였다. 이상의 결과들을 종합해 보면, 아연 자체가 A1 성상세포를 유도하는 역할을 할 뿐만 아니라, 염증성 자극에 의한 A1 성상세포 유도에도 아연이 관여하는 것으로 생각된다.

본 연구는 A1 성상세포 유도의 다양한 기전을 밝히고자 하는 시도로, 아연의 역할을 조명한 첫 연구이다. A1 성상세포 유도에서의 아연의 역할을 이해하는 것은 향후 여러 신경계 질환에서 신경손상의 기전을 밝히고, 치료제를 개발하는 데 큰 도움을 줄 것으로 기대한다.