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Research paper

Citrullinated isomer of myelin basic protein can induce inflammatory responses in astrocytes

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ABSTRACT

Purpose: During the course of demyelinating inflammatory diseases, myelin-derived proteins, including myelin basic protein(MBP), are secreted into extracellular space. MBP shows extensive post-translational modifications, including deimination/citrullination. Deiminated MBP is structurally less ordered, susceptible to proteolytic attack, and more immunogenic than unmodified MBP. This study investigated the effect of the deiminated/citrullinated isomer of MBP(C8) and the unmodified isomer of MBP(C1) on cultured primary astrocytes.

Methods: MBP charge isomers were isolated/purified from bovine brain. Primary astrocyte cultures were prepared from the 2-day-old Wistar rats. For evaluation of glutamate release/uptake a Fluorimetric glutamate assay was used. Expression of peroxisome proliferator-activated receptor-gamma(PPAR-γ), excitatory amino acid transporter 2(EAAT2), the inhibitor of the nuclear factor kappa-B(ikB) and high mobility group-B1(HMGB1) protein were assayed by Western blot analysis. IL-17A expression was determined in cell medium by ELISA.

Results: We found that MBP(C8) and MBP(C1) acted differently on the uptake/release of glutamate in astrocytes: C1 increased glutamate uptake and did not change its release, whereas C8 decreased glutamate release but did not change its uptake. Both isomers increased the expression of PPAR-γ and EAAT2 to the same degree. Western blots of cell lysates revealed decreased expression of ikB and increased expression of HMGB1 proteins after treatment of astrocytes by C8. Moreover, C8-treated cells released more nitric oxide and proinflammatory IL-17A than C1-treated cells.

Conclusions: These data suggest that the most immunogenic deiminated isomer C8, in parallel to the decreases in glutamate release, elicits an inflammatory response and enhances the secretion of proinflammatory molecules via activation of nuclear factor kappa B(NF-kB).

Summary statement: The most modified-citrullinated myelin basic protein charge isomer decreases glutamate release, elicits an inflammatory response and enhances the secretion of proinflammatory molecules via activation of nuclear factor kappa B in astrocytes.

1. Introduction

Astrocytes regulate essential functions to maintain homeostasis of the CNS. During CNS injury, infection, and inflammation, astrocytes produce a wide range of proinflammatory factors, including chemokines and cytokines, and increase the expression of innate immune receptors and molecules, including MHC-II. (Kiray et al., 2016; Li et al., 2016). Astrocytes also produce anti-inflammatory cytokines, heat shock proteins, and neuroprotective factors that participate in the regeneration and remyelination of neurons. (Gaudet and Fonken, 2018). The

Abbreviations: bFGF, basic fibroblast growth factor; CNS, the central nervous system; DAMPs, damage-associated molecular patterns; EAAT, excitatory amino acid transporter; ER, endoplasmic reticulum; HBSS, Hank's balanced salt solution; HMGB1, high mobility group box 1; IkB, inhibitor of nuclear factor kappa B; iNOS, inducible nitric oxide synthase; IP3, inositol-1,4,5-trisphosphate; LRP1, low-density lipoprotein receptor-related protein; MBP, myelin basic protein; NF-κB, nuclear factor kappa B; PPAR-γ, peroxisome proliferator-activated receptor gamma; TK, Tyrosine Kinase.

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metabolic interactions between neurons and astrocytes are well known; however, little is known about the cross-talk between astrocytes and oligodendrocytes, the myelinating cells of the CNS. This communication occurs via direct cell–cell contact as well as via secreted cytokines, chemokines, exosomes, and signaling molecules (Nutma et al., 2020).

Astrocytes are the lead players in the regulation of extracellular glutamate. The presence of high-affinity glutamate transporters on astrocytes represents the primary mechanism for the removal of glutamate from the extracellular space and protects the brain from glutamate-induced overexcitation of neurons (Schousboe et al., 2014). Excitotoxicity is a relevant mechanism of cell death in many disorders, and astrocyte–oligodendrocyte cross-talk plays an essential role in glutamate-dependent immunopathogenesis. Oligodendrocytes are sensitive to excitotoxic damage, and a high concentration of glutamate in the extracellular space induces the death of oligodendrocytes, leading to the initiation of demyelinating diseases (Matute et al., 2001). Glutamate release or increased activity of EAATs in astrocytes could thus be a relevant mechanism of treatment in diseases involving glutamate-mediated cytotoxicity. On the other hand, glutamate can also promote oligodendrocyte differentiation by activating various glutamate receptors (Tsitsilashvili et al., 2018), and it can also regulate the remyelination process (Wheeler and Fuss, 2016). Thus, variation in astrocyte-dependent release and removal of extracellular glutamate may be essential in oligodendrocyte homeostasis.

Recent investigations of ours showed that extracellular glutamate might participate in the plasticity of macrophages by inducing the expression of PPAR-γ and EAAT2 (Shanshiashvili et al., 2017). The increased intracellular concentration of glutamate can lead to the rearrangement of oxidative metabolism and accelerate the plasticity of macrophages toward immunosuppressive phenotypes. Microglia and astrocytes are critical in the development of neuroinflammatory diseases (Fakhoury, 2018; Palpagama et al., 2019), suggesting that regulation of PPAR-γ and EAAT2 can play an important role in attenuating neurodegenerative processes in these cells (Bordet et al., 2016).

MBP, a protein with a molecular weight of 18.5 kDa, is a significant component of CNS myelin (Shanshiashvili and Mikeladze, 2003). The structure of MBP is highly variable due to alternative mRNA splicing and post-translational modifications (Boggs, 2006). MBP and its fragments are secreted into extracellular space during the course of demyelinating diseases and after damage to the myelin sheath. MBP and MBP-derived peptides are well-known nonspecific ligands that weakly associate with many cellular proteins. Stapulionis et al. showed that in macrophages, MBP is a potent and specific ligand for αMβ2-integrin (Mac 1, CD11b/CD18) (Stapulionis et al., 2008), which is expressed predominantly in myeloid cells and which mediates the adhesive reactions of leukocytes during the inflammatory response (Prince et al., 2001). MBP can bind to LRP1 in astrocytes and is implicated in myelin phagocytosis (Gaultier et al., 2008). Significantly increased (α2M-R/LRP) immunoreactivity was identified in reactive astrocytes, indicating that expression of this receptor is regulated in vivo in response to brain injury (Lopes et al., 1994). Besides, as it is a cationic protein, MBP acts on the receptor of the basic fibroblast growth factor (bFGF) and promotes astrocytogenesis (Duong et al., 2019).

MBP shows extensive post-translational modifications, including the deimination (citrullination) of arginine residues. Deiminated MBP is structurally less ordered and more susceptible to proteolytic attack than the unmodified form. The reduced cationicity of deiminated MBP impedes membrane assembly (Shanshiashvili et al., 2014) and exposes an immunodominant epitope in the membrane-bound protein to proteases. This exposure may then cause the deiminated, highly immunogenic epitope to be released, priming the innate cells of the CNS. Deiminated MBP, the most immunogenic isomer of MBP, elicits an inflammatory response of astrocytes in demyelinating diseases and enhances the ability of astrocytes to release proinflammatory mediators (Moscarello et al., 2007). In a previous study (Shanshiashvili et al., 2017), we found that the deiminated isomer of MBP (C8) tends to polarize RAW 264.7 macrophages into proinflammatory M1 phenotypes, whereas the unmodified isomer (C1) enhances the activity of M2 phenotype markers. C8 differs from C1 by deimination of 6–11 arginines to form citrullines (Wood and Moscarello, 1989). Given that these isomers of MBP can be formed during myelin damage, we proposed that they act differently on astrocyte activity.

Astrocytes detect injury and infection signals from oligodendrocytes, responding by secreting proinflammatory cytokines, including TNF-a, IL-6, IL-17A (Soung and Klein, 2020). Overproduction of IL-17A promotes inflammation in autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, psoriasis, etc (Weaver and Murphy, 2007). Recent studies have shown that, besides inflammatory actions, IL-17A could promote glutamate excitotoxicity by decreasing astrocyte ability to uptake glutamate and stimulating Ca2 + -dependent glutamate release (Kostic et al., 2017). These data suggest that IL-17A in astrocytes contributes to glutamate excitotoxicity, which provides a link between inflammatory and neurodegenerative processes in CNS. Various transcription factors, including NF-kB, regulate IL-17A gene expression. Thus, modulation of the transcriptional regulation of IL-17A expression is one of the approaches for the treatment of autoimmune diseases (Khan and Ansar Ahmed, 2015).

In this study, we show that the deiminated isomer of MBP, unlike the unmodified protein, decreased glutamate release and induced secretion of nitric oxide and IL-17A, probably due to a change in NF-kB and PPAR-γ-dependent transcription.

2. Material and methods

2.1. MBP isolation and purification

MBP isomers (C1 and C8) were isolated and purified from bovine brain white matter according to the method of Chou et al. (Chou et al., 1976) with slight modifications (Shanshiashvili et al., 2003). SDS-PAGE was used to verify the purity of the isomers. They were lyophilized and stored until use at –20 °C.

2.2. Rat primary astrocyte cultures

Primary astrocyte cultures were prepared from the whole brains of 2-day-old Wistar rats. Pups were sacrificed by decapitation; brains were then harvested and placed in 100 mm Petri dishes containing PBS. Brain tissue was dissociated, followed by trituration in 10 mL DMEM/F12 medium (ATCC, Manassas, VA, USA) containing 10% deactivated fetal bovine serum (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany), 100 U/mL penicillin (Gibco® by Life Technologies), and 100 μg/mL streptomycin (Gibco®). Dissociated tissue was passed through a 70 μm cell strainer to remove tissue debris. The initial mixture of dissociated glial cells was seeded in 75 cm^3 flasks coated with poly-D-lysine (Sigma-Aldrich). The medium was changed every 2–3 days to wash out cells other than glial cells. For isolation of microglial cells, confluent cultures between days 12 and 14 were agitated on a rotary shaker at 220 rpm and 37 °C for one h. Floating cells were removed from the dishes. After 21 days, the astrocytes— cells remaining adherent after the removal of microglia—were removed from the flasks by incubation with 0.25% trypsin (Carbosynth, Compton Berkshire, UK) for 10 min. After being washed in PBS, cells were ready for seeding and experimental use. Isolated astrocytes were determined to be > 95% pure based on morphological characteristics.

2.3. Incubation of cells with MBP

After 21 days, primary astrocytes were incubated for 24 h in serum-free DMEM in a 24-well plate at 37 °C in a humidified atmosphere of 95% air and 5% CO2. After 24 h, cells were used in the glutamate uptake and release assay in the presence and absence of 0.5 μM MBP charge isomers.

2.4. Western blotting

Following incubation in the presence or absence of MBP charge isomers (C1 or C8, 0.5 M), cells were subjected to Western blotting according to the method described in the initial study.[22] The following primary antibodies were used: anti-EAAT-2 (ab41621, Abcam), anti-HMGB1 (ab18256, Abcam), anti-PPAR-γ (ab191407, Abcam), anti-ikB (sc-371, Santa Cruz Biotechnology, USA). Immunoreactivity was detected by enhanced chemiluminescence autoradiography (ECL kit, Santa-Cruz Biotechnology). Protein concentrations were determined using a BCA protein assay kit (Thermo Scientific). Signal quantification from Western blots was assisted by ImageJ software.

2.5. Glutamate uptake and release assay

The assay was carried out according to Mahmoud et al. (Mahmoud et al., 2019a) 100,000 astrocytes were seeded in each well of a 24-well plate washed twice with HBSS (Gibco, Grand Island, NY, USA) containing Ca^{2+} (1.26 mM $CaCl_2$) at pH 7.25. For glutamate uptake, cells were incubated with 100 μM glutamate in the HBSS with Ca^{2+} for 4 h, while for glutamate release, astrocytes were incubated in HBSS with Ca^{2+} without glutamate for 2 h at 37 °C with 5% CO2. Then, the culture medium was collected and transferred to a solid black 96-well microplate. Glutamate uptake by astrocytes was measured by subtracting the amount of glutamate measured in the medium from the amount initially added to the cells.

2.6. Glutamate detection

A Fluorimetric glutamate assay kit (Abcam, Cambridge, UK) was used to measure glutamate according to the manufacturer's instructions, monitoring fluorescence with a Twinkle LB970 plate reader (Berthold Technologies) with excitation at 530 nm and emission at 570 nm. Data are expressed as the mean ± SEM of triplicate wells. The amounts of glutamate were measured at the start and end points of incubation in the medium and then were compared for each sample. To determine glutamate concentration in the samples, we constructed a standard curve using cell-free media containing a known concentration of glutamate. Untreated (i.e., without MBP) cells were used as controls in both uptake and release experiments. Glutamate uptake and release by astrocytes were normalized to the amount of protein in the corresponding cell medium, measured using a micro BCA protein assay kit (Thermo Scientific, Rockford, IL, USA).

2.7. Cytokine – IL17A detection

The level of IL-17A in the supernatants of primary astrocytes were measured by ELISA according to the manufacturer's protocol (ab21402, Abcam).

2.8. Nitrite assays

Nitrite accumulation was used as an index of NO production and inflammatory activation in general. The assay was carried out as in our previous works (Shanshiashvili et al., 2012). The widely used bacterial antigen LPS (a proinflammatory agent) was used to evaluate the inflammation rate in cultured astrocytes. Fresh culture medium served as the blank in all experiments. The concentration of LPS was 100 ng/mL, and incubation times were 24, 48, and 72 h.

2.9. Statistical analysis

All data are presented as mean ± SEM. Statistical analysis used one-way ANOVA, followed by Scheffe's post hoc comparison test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of C1 and C8 on glutamate uptake and release from astrocytes

Taking into account that astrocytes maintain glutamate homeostasis in the CNS by controlling the balance between glutamate uptake and release (Mahmoud et al., 2019b), the action of MBP isomers on the release and uptake of glutamate was investigated. Two natural charge isomers of the classic 18.5 kDa MBP, C1 and C8, were used in these experiments. C8 is a major deiminated (citrullinated) isomer that differs from C1 by deimination of 6–11 arginines to citrulline (Wood and Moscarello, 1989). After incubation with MBP isomers, the release and uptake of glutamate by astrocytes were determined. We found that the unmodified isomer, C1, did not change the release of glutamate but increased glutamate uptake by astrocytes (Fig. 1). On the contrary, the C8 deiminated (citrullinated) isomer did not significantly alter uptake but decreased glutamate release. These data suggest that the unmodified C1, unlike citrullinated C8, accelerates the accumulation of glutamate by astrocytes.

3.2. Effects of C1 and C8 on EAAT2, PPAR-γ, IkB, and HMGB1 expression in astrocytes

Uptake and release of glutamate by astrocytes occur by different mechanisms. Astrocytic glutamate release is mainly mediated by Ca^{2+}-dependent exocytosis, while Na^{+}-dependent EAAT-2/GLT-1 transporters carry out glutamate uptake. The expression of the EAAT-2 transporter positively correlates with synaptic activity and is upregulated with increased glutamate release from the surrounding glutamatergic neurons (Gegelashvili and Schousboe, 1997; Schlag et al., 1998; Swanson et al., 1997). To determine whether these effects of the MBP isomer are the result of EAAT-2 upregulation, we analyzed the expression of EAAT-2 in astrocytes. Our results show that both isomers equally increased the level of EAAT-2 (Fig. 2 A, B).

Glutamine/glutamate metabolism changes the activity of several transcription factors, including PPAR-γ (Ban et al., 2011). The PPAR family comprises transcriptional factors involved in the regulation of lipid and glucose metabolism and cell differentiation and proliferation. Among other effects, activation of PPAR-γ has a protective effect on the excitotoxicity of glutamate by increasing EAAT-2 expression in astrocytes (Omeragic et al., 2017). Based on this observation, we proposed that PPAR-γ may be involved in the MBP-dependent expression of EAAT-2. To test this hypothesis, we determined the expression level of PPAR-γ in MBP-treated astrocytes. We found that both MBP isomers equally increased the expression of PPAR-γ (Fig. 2A, C), and there was no difference between the action of the modified and unmodified forms of the protein. These data suggest that the binding partner of MBP in astrocytes, which regulates the activity of PPAR-γ, cannot recognize various isomers of MBP, and the different effects of these proteins on the absorption and release of glutamate occur in another way. However, because both MBP isomers equally increase PPAR-γ expression, it suggests that these proteins can bind to various receptors on astrocytes, and their activities are more complex.

Another transcription factor that can regulate expression of the EAAE-2 gene is NF-κB, which is a crucial regulator of EAAT2 expression in response to diverse cellular signals (Martinez-Lozada et al., 2016). Astrocytes were shown to directly clear myelin debris in vitro and in vivo after neuronal injury (Wang et al., 2020). Phagocytosis of myelin debris by astrocytes early after damage leads to NF-κB signaling and chemokine secretion (Claycomb et al., 2013; Ponath et al., 2017). The receptor of FGF, one of the targets of MBP, as well as the LRP-1, another possible binding partner of MBP, can regulate glutamate homeostasis in astrocytes via the ERK/NF-kB pathway (Swanson et al., 1997; Suzuki et al., 2001; Ghosh et al., 2011). Furthermore, plasma membrane pattern recognition receptors (PRRs) expressed in astrocytes play an essential

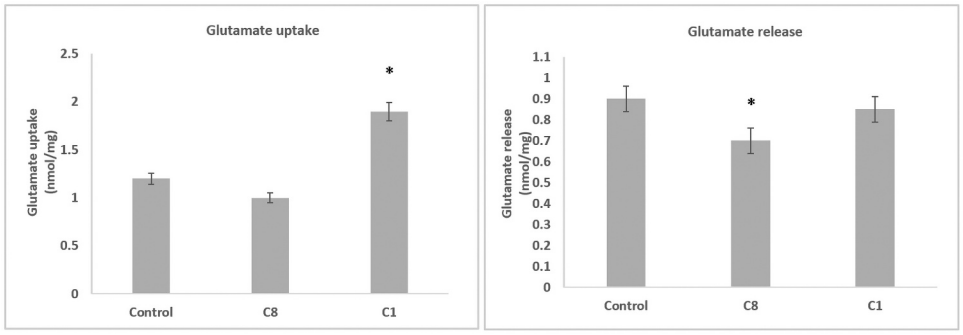

Fig. 1. Alteration of glutamate uptake and release in astrocytes after treatment of cells by C8 and C1. Data represent mean ± SEM of results from three separate experiments in duplicate. * denotes $P < 0.05$ vs. control (untreated) cells.

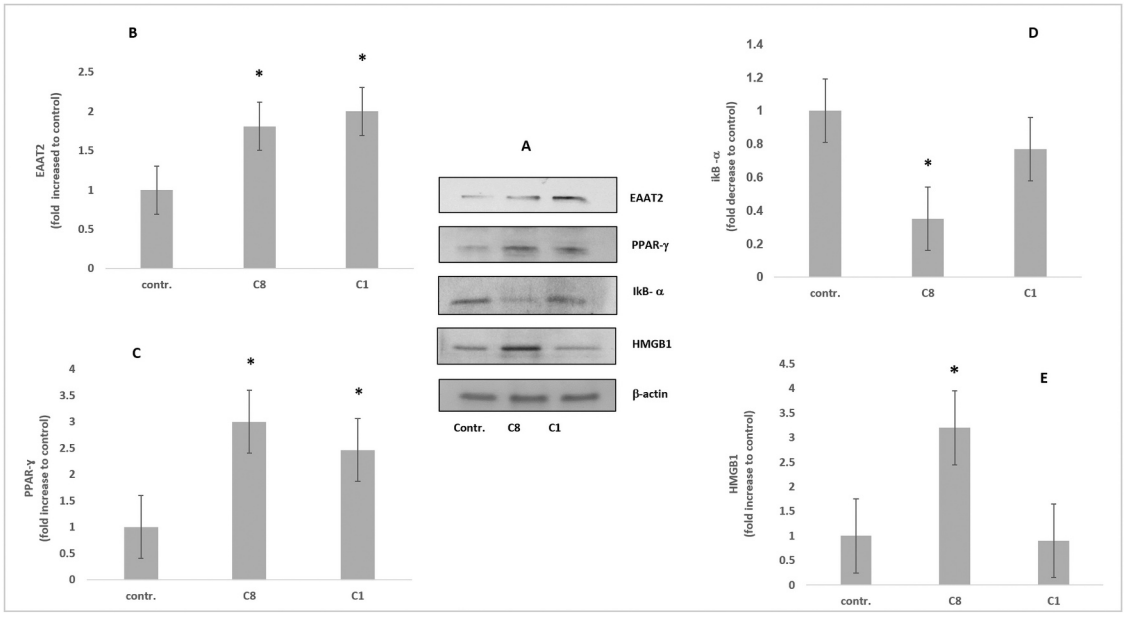

Fig. 2. Mean values and representative Western blots of EAAT-2, PPAR- γ, IkB and HMGB1 in astrocytes after treatment of cells by C8 and C1. Astrocytes (5×10^5 cells per well) were incubated with C1 and C8 isomers (0.5 μM) for 24 h, followed by the determination of EAAT-2, PPAR- γ, IkB and HMGB1 expression by Western blot analysis. The data shown are representative of three independent experiments. β-Actin was also visualized by Western blotting to confirm the equal loading of the fractions. Quantification of EAAT2 blots is shown in B, of PPAR-γ in C, of IkB in D, and of HMGB1 in E. In each experiment * denotes $P < 0.05$, vs. the corresponding control.

role in the activation of NF-κB and mitogen-activated protein kinase inflammatory pathways (Heneka et al., 2014). Thus, MBP isomers can act on glutamate homeostasis by activating NF-κB via these receptor systems. To determine whether this proinflammatory transcription factor is involved in the effects of MBP isomers, we determined by Western blotting the expression level of IkB, an inhibitor of NF-κB, the signal-induced degradation of which initiates canonical activation of NF-κB (Liu et al., 2017). Our results show that treating cells with C8 decreased IkB (Fig. 2A, D), whereas the unmodified C1 did not change the level of this protein. The data show that C8 increased NF-κB activity by inducing the degradation of inhibitory protein-IkB. Thus, it can be assumed that NF-κB, one of the main transcriptional factors responsible for inflammation, is activated by deiminated MBP.

Several inflammatory response factors, including alarmins or DAMPs, become upregulated during neuroinflammation. Alarmins are released upon tissue damage and contribute to the dysregulation of inflammatory and autoimmune processes, as well as the orchestration of tissue homeostasis, including repair and remodeling. HMGB-1, an important DAMP, can promote neuroinflammation by facilitating the formation of the NLRP3 inflammasome in astrocytes and microglia (Yao et al., 2019; Kim et al., 2008). HMGB1 can effectively promote NLRP3 inflammasome formation in astrocytes by activating NF-κB. Interestingly, IL4 was shown to inhibit NLRP3 inflammasome formation through negative regulation of NF-κB and promotion of PPAR-γ activation (Yao et al., 2019). Thus, we next determined the production of HMGB1. Western blots of cell lysates showed increased intracellular levels of HMGB1 in the presence of C8, whereas C1 did not change the production of HMGB1 (Fig. 2A, E).

3.3. *Effects of C1 and C8 on NO and IL-17A secretion in astrocytes*

To confirm the observation that NF-κB can participate in the effects of citrullinated MBP, additionally, we determined the amount of nitric oxide. Induction of inducible nitric oxide synthase (iNOS), NO, and NO-related byproducts has been found in demyelinating conditions, and NO can play a distinct, detrimental, or protective role during different stages of inflammation (Arnett et al., 2002). The induction of iNOS through inflammatory pathways is mainly dependent on NF-κB activity (Aktan, 2004). After 24, 48 and 72 h of exposure of primary astrocytes to 0.5 μM of C1 or C8, we determined the production of nitric oxide (Fig. 3).

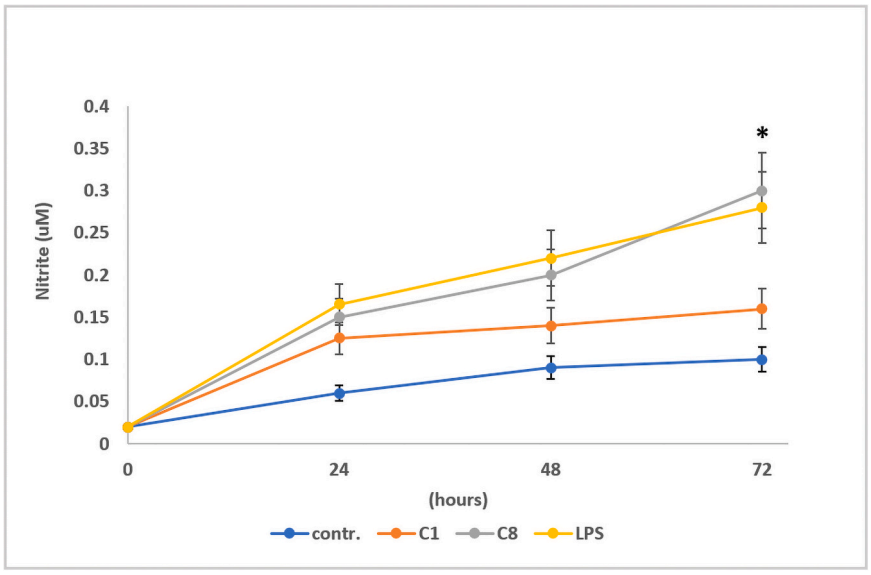

Fig. 3. Effect of LPS, C8 and C1 isomers on NO secretion in astrocytes. Astrocytes (5×10^5 cells per well) were incubated with LPS (100 ng/mL), and C1 and C8 isomers (0.5 μM) for 24, 48 and 72 h, followed by determination of nitrite concentration in cell culture medium. Data are represented as mean ± SEM of results from four separate experiments performed in duplicate. In each experiment * denotes $P < 0.05$ (LPS, C8) vs. the corresponding control (72 h).

We found that C8 increased nitric oxide synthesis in all time intervals and the amount of secreted NO was comparable to the amount of nitric oxide produced in the presence of LPS. These results suggest that deiminated MBP, in contrast to the unmodified form, can induce the secretion of inflammatory molecules through the activation of NF-κB signaling.

One of the significant cytokines that are released by astrocytes during inflammation is IL-17. This family of cytokines has been linked to many autoimmune-related diseases, including rheumatoid arthritis, asthma, and CNS disorders. Taking into account that the metabolic response to inflammation in astrocytes is regulated by nuclear NF-κB signaling (Robb et al., 2020) and that glutamate supplementation can induce acceleration of mitochondrial metabolism via this transcription factor; we determined the secretion of IL-17A in the presence and absence of glutamate. For this, we treated astrocytes with 0.1 mM glutamate and analyzed the release of IL-17A after 4 h. Our results showed that only in the presence of glutamate and deiminated C8 astrocytes secreted IL-17A (Fig. 4).

Thus, activation of glutamate uptake and subsequent acceleration of NF-κB signaling can change the expression of IL-17A by astrocytes (Khan and Ansar Ahmed, 2015). These data support the suggestion that astrocytes respond to the action of deiminated MBP as an inflammatory signal and increase the activity of NF-κB, with subsequent production and secretion of inflammatory molecules, including HMGB protein, NO and IL-17A.

4. Discussion

During the active stages of the immune response, infiltrating cells and endogenous activated microglia, astrocytes, and oligodendrocytes

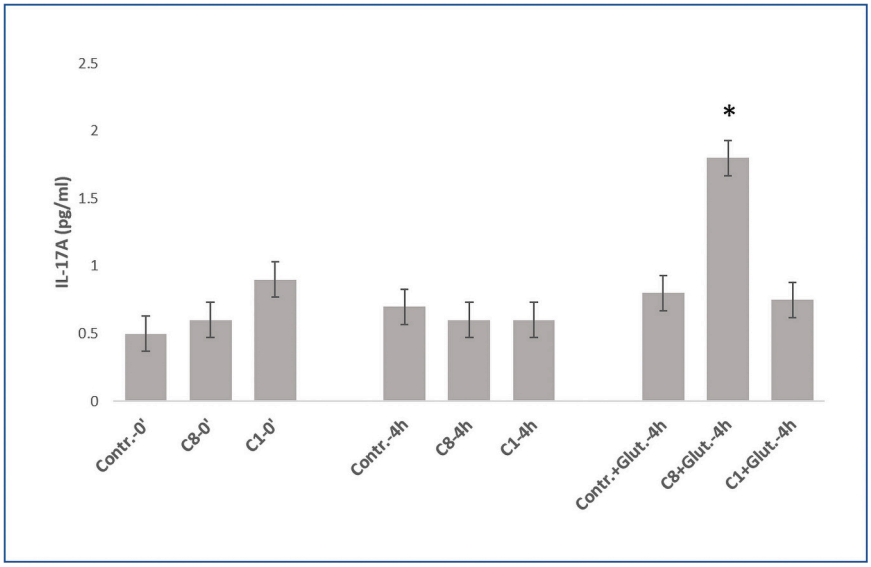

Fig. 4. Interleukin − 17A production by the astrocytes under the stimulation with MBP isomers (C1, C8) in the presence and absence of glutamate. Data are represented as mean ± SEM of results from four separate experiments performed in duplicate. In each experiment * denotes $P < 0.05$ (C8) vs. the corresponding control.

release multiple factors that can change the duration of disease and protect intact tissue from further damage. MBP is one of the main autoantigens of the myelin sheath, being implicated in autoimmune neurological disorders (Lutton et al., 2004; Sospedra and Martin, 2005). Post-translational modifications of MBP generate charge micro-heterogeneity of protein that can play an important role in the etiopathogenesis of demyelinating diseases (Kim et al., 2003; Wood and Moscarello, 1997; Harauz et al., 2004). These charge isomers are C1–C8: C1 is the least modified and most cationic, and C8 is the most modified and least cationic (Harauz et al., 2004; Fannon and Moscarello, 1991). The C1 isomer is N-terminal acylated and potentially methylated on a specific arginyl residue and carries a high net positive charge of + 19 at neutral pH, whereas in C8 are generally six deiminated arginines and carries a reduced net positive charge of + 13 at neutral pH. The severity of multiple sclerosis (MS) is correlated with the degree of deimination (citrullination) of MBP (Harauz and Musse, 2007). Unlike other isomers, C8 is predominant in loosely compacted myelin because the deimination of arginine in C8 significantly influences the biophysical structure and functional properties of MBP (Wang et al., 2011). Using optical waveguide lightmode spectroscopy (OWLS), we previously showed (Shanshiashvili et al., 2014; Shanshiashvili et al., 2003) that different charge isomers of MBP interact differently with model membranes in terms of their association rate coefficient, adhesion, conformation and clustering tendency. The C1-charged isomer is the most active, and C8 is the least active in absorption into the myelin membrane. In addition, C8 is characterized by higher immunogenicity than C1 (Tranquill et al., 2000). Interestingly the deiminated C8 isomer of MBP tends to polarize RAW macrophages into M1 phenotypes, whereas C1 enhances the activity of M2 phenotype markers (Tsitsilashvili et al., 2019).

In this study, we investigated the effect of C1 and C8 isomers on some parameters of astrocyte activity. Considering that in demyelinated pathologies, the myelin sheath breaks down, and MBP and its fragments are secreted into the extracellular fluid, we hypothesized that the action of MBP could play a significant role in oligodendrocyte–astrocyte interactions. Several studies have highlighted the cross-talk between astrocytes and oligodendrocytes (ELBini and Neili, 2023). Three binding partners can interact with MBP in astrocytes: bFGF, LRP, and PRRs. All three partners, directly or indirectly, can mediate the action of MBP via various pro- and anti-inflammatory transcription factors, including NF-κB (Swanson et al., 1997; Suzuki et al., 2001; Ghosh et al., 2011; Heneka et al., 2014). We propose that one of the targets of MBP in astrocytes is the adhesion molecule LRP, which after cleavage by MBP is able to activate PPAR-γ (Lutz et al., 2016). Our results show that both MBP isomers enhance the expression of PPAR-γ, suggesting that this acceleration does not depend on the electrostatic charge of the MBP. Nevertheless, it can be concluded that one of the acceptors of MBP in astrocytes is the cell adhesion molecule L1. Apparently, this activation is a compensatory process and is directed to neurorestorative actions in neurons (Yan et al., 2021). However, since MBP isomers alter the secretion of inflammatory mediators in different ways, it can be concluded that other receptor systems are also involved in the effects of MBP isomers. These may be systems involving NF-κB. Evidently, deletion or disruption of significant proinflammatory NF-κB signaling pathway in astrocytes diminishes the recruitment of inflammatory cells after autoimmune disease, and the inhibition of NF-κB in astrocytes results in neuroprotective effects following experimental autoimmune encephalomyelitis (Brambilla et al., 2009). From our results, we infer that treating astrocytes with C8 increases NF-κB activity, whereas C1 does not change its expression. The results obtained in the analysis of NO, IL-17A and intracellular levels of HMGB1 protein agree with these observations: only treatment of astrocytes with C8 significantly increased HMGB1 protein expression and NO production. Since NF-κB is one of the main inducers of iNOS and HMGB1, and excess NO and HMGB1 production aggravates neuronal damage in neurodegenerative diseases (Calabrese et al., 2007; Paudela et al., 2019), we propose that the deiminated isomer of MBP enhances the proinflammatory response of astrocytes. Astrocytes maintain glutamate homeostasis in the CNS by controlling the balance between glutamate uptake and release. Accordingly, astrocytes can also support normal neuronal function and protect against glutamate excitotoxicity (Mahmoud et al., 2019b). The uptake and release of glutamate by astrocytes occur in different systems. Astrocytic glutamate transporter EAAT2 (GLT-1) is a critical component in the regulation of neuronal transmission and is most commonly associated with excitatory synapses. This Na-dependent transporter is responsible for the majority of extracellular glutamate clearance from the synaptic cleft and is essential for excitatory neurotransmitter balance (Tanaka et al., 1997).

On the other hand, glutamate is released from astrocytes by Ca-dependent exocytosis (Cornell-Bell et al., 1990; Pasti et al., 1997). Glutamate released from astrocytes is involved in the regulation of neuronal activity under physiological conditions (Hamilton and Attwell, 2010). Ca^{2+}-mediated exocytosis is thought to be the primary mechanism mediating glutamate release under physiological conditions (Bezzi et al., 2004). The majority of Ca^{2+} elevation necessary for glutamate release by astrocytes comes from the ER and, accordingly, is triggered by the formation of IP3. We found that MBP isomers act differently on the uptake and release of glutamate: the unmodified isomer of MBP (C1) increases glutamate uptake and does not change the release of glutamate by astrocytes, whereas the deiminated isomer (C8) decreases the release of glutamate by astrocytes but does not change the uptake. These data suggest that in the presence of C8, astrocytes release less glutamate than in the presence of C1, and contrariwise C1, unlike C8, increases glutamate uptake. These data can be explained by the supposition that MBP isomers act on various targets in astrocytes; C8 acts predominantly through inflammasomes, while the effect of C1 is apparently mediated by the TK-coupled (Tyrosine Kinase) basic fibroblast growth factor receptor (bFGF receptor) (Zelenaia et al., 2000).

5. Conclusion

The main conclusion of this study is that the deiminated, i.e. a citrullinated, isomer of MBP, elicits an inflammatory response in astrocytes. Taking into account that citrullinated MBP is less ordered than uncitrullinated MBP, and given that aggregated and misfolded proteins induce NLRP activation (Shi et al., 2015), it is proposed that the action of MBP isomers can be differentiated at the level of inflammasome formation. Additional studies are needed to determine the significance of the targeted effect of MBP isomers on astrocytes.

Ethics approval and informed consent

The study was approved by the Bioethics Commission of the I. Beritashvili Experimental Biomedicine Center (Tbilisi, Georgia; ref. N01/04.02.2021). All authors read and approved the manuscript.

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CRediT authorship contribution statement

D. Mikeladze and L. Shanshiashvili - (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, (3) final approval of the version to be submitted. M. Chikviladze, N. Mamulashvili, N. Narmania, M. Sepashvili - carried out the experimental work. J. Ramsden - (2) drafting the article or revising it critically for important intellectual content, (3) final approval of the version to be submitted. All authors contributed to giving final approval of the version to be published and agreed to be accountable for all

aspects of the work. D. Mikeladze and L. Shanshiashvili are co-senior authors contributing equally to this paper.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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Consent for publication

All the authors consent to publish this manuscript.

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