Research article

INCREASED EXPRESSION OF PAD2 AFTER REPEATED INTRACEREBROVENTRICULAR INFUSIONS OF SOLUBLE Aβ25-35 IN THE ALZHEIMER'S DISEASE MODEL RAT BRAIN: EFFECT OF MEMANTINE

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Abstract: Peptidylarginine deiminases (PADs) convert the arginine residues in proteins into citrulline residues in a Ca^{2+}-dependent manner. We previously showed that a bilateral injection of ibotenic acid into the rat nucleus basalis magnocellularis elevated the PAD2 activity in the hippocampus and striatum. In this study, we examined whether repeated intracerebroventricular infusions of soluble $Aβ_{25-35}$ would affect the PAD2 expression in any regions of the rat brain. We also assessed the protective effect of memantine on Aβ-induced PAD2 alterations. The infusion of $Aβ_{25-35}$ increased the activity and protein level of PAD2 in the hippocampus, and co-treatment with memantine suppressed these changes. An immunohistochemical analysis showed that an increased level of PAD2 was coincident with GFAP-positive astrocytes and CD11b-positive microglia. In addition, immunofluoresecence staining revealed that citrulline-postive immunoreactivity coincided with the occurrence of GFAP-positive astrocytes. Co-treatment with memantine reversed the activation of the astrocytes and microglia, thus attenuating the PAD2 increment. These biochemical and immunohistochemical results suggest that PAD2 might play an important role in the pathology of early Alzheimer's disease, and may correlate with the changes in glial cells that are recovered by memantine treatment.

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Abbreviations used: Aβ – amyloidβ; AD – Alzheimer's disease; GFAP – glial fibrillary acidic protein; PAD – peptidylarginine deiminase

INTRODUCTION

Peptidylarginine deiminases (PADs; protein-arginine deiminase, EC 3.5.3.15) are a group of enzymes that convert protein arginine residues to citrulline residues in a Ca^{2+}-dependent manner. In mammalian tissues, PADs occur as five different isoforms, i.e. types 1-4 and 6, which display nearly identical amino acid sequences [1, 2] but are dissimilar in terms of tissue-specific expression. PAD2 is abundantly present in the central nervous system (CNS) [3]. However, this isoform is also present in various other tissues, including the salivary and sweat glands, pancreas, skeletal muscle, uterus, spleen, skin and bone marrow [4]. Immunohistochemical studies demonstrate that PAD2 is localized in the glial cells, astrocytes and microglial cells and also in oligodendrocytes [5]. It is assumed that PAD2 normally remains inactive but becomes active and citrullinates cellular proteins when the intracellular calcium balance is upset during the early stage of neurodegenerative changes. In support of this notion, it has been reported that physiological insults such as hypoxia and kainic acid administration resulted in the activation of PAD2 and the appearance of citrullinated proteins, suggesting it as a marker of acute neurodegeneration [6, 7].

A large body of evidence indicates that amyloid β (Aβ) is the main component of senile plaques and it is considered to have a causal role in the onset and progress of Alzheimer's disease (AD) [8]. The mechanisms associated with Aβ toxicity are not clearly defined, but they appear to be associated with the production of free radicals and glutamate-mediated excitotoxicity. In support of this, there are reports that Aβ-induced neurodegeneration in the adult rat brain and in cultured cortical neurons is mediated by the activation of the N-methyl-D-aspartate (NMDA) receptor, and that treatment with the non-competitive low- to moderate-affinity NMDA receptor antagonist, memantine, had a protective effect [9, 10]. Moreover, amyloid-β peptide has been shown to elevate cytosolic Ca^{2+} levels in neurons, an action that has been proposed to be secondary to the generation of reactive oxygen species [11]. Since PAD2 activity is solely dependent on calcium ions, it might be involved in AD and other neurodegenerative disorders. In line with this possibility, we previously reported that infusing ibotenic acid into the nucleus basalis magnocellularis (NBM) caused the up-regulation of PAD2 activity and increased its immunoreactivity in the rat hippocampus and striatum [12]. Furthermore, Ishigami *et al.* [13] recently showed the abnormal accumulation of citrullinated proteins catalyzed by PAD2 in hippocampal extracts from patients with AD. However, the correlation between PAD2 and AD-related pathological changes is not clear. Therefore, in this study, we investigated the effects of repeated intracerebroventricular infusions of soluble $Aβ_{25-35}$ and of the concomitant administration of memantine on PAD2 in certain regions of the AD model rat brain.

MATERIALS AND METHODS

Animal surgery and drug administration

Male Wistar rats (Sankyo Laboratory, Tokyo, Japan, 9-10 weeks old, body weight 280-300 g) were housed under standard environmental conditions (12 h light/dark cycle, 22ºC) and allowed free access to food and water for two weeks prior to the treatment. All the animal experiments were carried out according to the Japanese Animal Experiment Guidelines, 1980, and were approved by the Animal Experiment Committee of Yokohama City University Graduate School of Integrated Science (YCU20409). $A\beta_{25-35}$ was dissolved initially in autoclaved Milli Q water, and finally at a concentration of 3.5 mM with PBS solution (vehicle). The dose of β-amyloid protein was selected on the basis of a previous study in which this peptide reduced synaptic transmission in the rat hippocampus [14]. After being anaesthetized with sodium pentobarbital (50 mg/kg, i.p.), the rats were placed in a stereotaxic instrument. Guide cannulas (BAS, Tokyo, Japan) were implanted into both ventricles under aseptic conditions through holes drilled in the skull at the following coordinates: anterior-posterior = -0.3, medial-lateral = +1.1, and dorsal-ventral = 3.6 mm from the bregma according to the atlas of Paxinos & Watson. Two days after the cannula implantation, 5 µl of $A\beta_{25-35}$ was injected into the ventricles sequentially over 5 min with a 5 µl Hamilton syringe through the cannula tubing for three consecutive days with light ether anesthesia. The needle was left in place for 5 min after the injection before being withdrawn. The control rats were infused with 5 µl PBS using the same procedure. 3 hours after the operation and continuing for 5 days thereafter, the rats were injected once a day with an intraperitoneal injection of either memantine (5 mg/kg per day) or saline (0.9% NaCl). The rats were rapidly decapitated 2 days after the last Aβ infusion, and their brain tissues were quickly collected and put on ice, and stored at -80ºC. The tissues were homogenized in 9 volumes of 20 mM Tris-HCl buffer (pH 7.4) and centrifuged at 100,000 g for 60 min at 4ºC. The supernatant was collected, and then stored at -80ºC until needed for the measurement of enzyme activities and Western blot analysis. A separate group of rats was prepared for the immunohistochemical analysis.

PAD activity and Western blot analysis

The PAD activity was measured using the fluorometric HPLC method as described previously [12]. Since the brain homogenate might contain the PAD4 isoform (derived from either the blood leucocytes [15] or the white matter [16]), the PAD2 activity is expressed as PAD2-like activity. To determine the quantitative protein level of PAD2, protein (10 µg) was fractionated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose membrane (Schleicher and Schuell Bioscience, Germany). The equal transfer of protein to the membrane was further verified by Ponceau S staining. The membrane was then probed with a rabbit anti-rat PAD2 antibody

(1:10000) [1], followed by incubation with a horseradish peroxidase-conjugated second antibody. The protein bands were detected via ECL and with a Western blotting detection kit (Amersham Biosciences) using the chemiluminescence system of the Image Reader LAS-1000 Plus software of Luminescence Image Analyzer (Fujifilm, Japan). The band intensity (in arbitrary units) was measured using Image Gauge software 4.0 (Fujifilm, Japan).

Immunohistochemistry
The immunohistochemical analysis was carried out as described previously [17]. 20-μm thick frozen sections of the brains were used for immunohistochemical staining with rabbit anti-cow GFAP antibody (Dako Glostrup, Denmark) (1:300), and anti-rat PAD2 antibody (1:1000) [18]. The specificity of the PAD2 antibody was evaluated via an immunoblot analysis where it did not cross react with PAD4 (data not shown); this specificity was already described in several studies [1, 12, 13, 15]. Hydrogen peroxide (0.3%) was used to quench the endogenous peroxidase activity. Non-specific binding was blocked by incubation in 5% normal goat serum for 30 min, followed by primary antibody incubation at 4ºC overnight. Biotinylated goat anti-rabbit (Vector Laboratories, U.S.A) IgG (H+L) was applied as the second antibody, and avidin-biotin-peroxidase complex (ABC kit, Vector laboratories, USA) served as the third reagent. The bound antibodies were then detected using a DAB kit according to the manufacturer's instructions (Dako Glostrup, Denmark). For double staining, the sections were incubated with mouse anti-porcine GFAP (1:300; Chemicon International, CA) with rabbit anti-rat PAD2 (1:1000). Deiminated proteins were stained with rabbit deiminated antibody as described previously [19] coupled with mouse anti-porcine GFAP (1:300). After washing with PBS, they were stained for 1 h with a combination of Cy2-congugated anti-rabbit IgG (1:100) and Cy3-labeled anti-mouse IgG (1:200; Jackson Immunoresearch Laboratories, Inc., USA). They were rinsed in PBS, mounted with Fluoromount G (Electron Microscopy Technique, USA), and examined under a fluorescent microscope (Axioplus, Carl Ziess, Germany). For the quantitative analysis of the GFAP-positive astrocyte, CD11b-positive microglia and Nissl-positive neurons in the pyramidal layer of the CA1 subfield of the hippocampus sections were counted (n = 3-4 rats) separately using an image analysis system (ImagePro-Axioplan). Using the 20x objective, the images were captured and digitalized. Cell counts were made within a field which was outlined with a calibration of 1300 x 1030 pixels of the tissue area. The average number of cells was calculated for 4 fields per section and 3-4 sections per animal.

Data analysis
One-way analysis of variance (ANOVA) was used for the statistical evaluation in all the experiments. The Bonferroni posthoc test was performed for multiple group comparisons. The data is shown as the means ± S.E.M. A value of $P < 0.05$ was considered statistically significant.

RESULTS

The effect of Aβ on PAD2 expression

To examine the possible effect of Aβ on PAD2, we measured the PAD2 enzyme activity in the hippocampus, frontal cortex and striatum after repeated intracerebroventricular infusions of soluble $Aβ_{25-35}$ and co-treatment with memantine. As shown in Fig. 1A, the activity of PAD2 increased 1.6-fold after Aβ infusion as compared with controls, whereas the activity in the frontal cortex and striatum did not change. To clarify whether increased PAD2 activity coincides with increased expression of its protein, we carried out an immunoblot analysis with a PAD2-specific antibody and found that PAD2 peptide expression significantly increased in the hippocampus ($P < 0.05$; Fig. 1B, C). Co-treatment with memantine partially suppressed the increase in PAD2 activity and immunoreactivity in the Aβ-treated hippocampus (Fig. 1). Moreover, an immunohistochemical analysis showed that treatment with Aβ significantly increased the PAD2 level, although a considerable immunoreactivity was also evident in the control hippocampus (Fig. 2A). Concomitant administration of memantine attenuated the Aβ-induced activation of PAD2 expression in the hippocampus (Fig. 2).
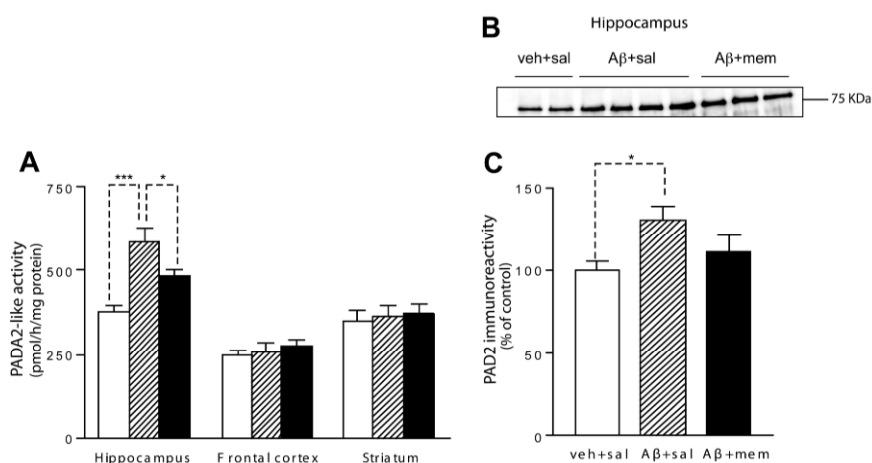

Fig. 1. PAD2 expression in three regions of the rat brain after treatment with Aβ alone or with Aβ and memantine. The enzyme activity of PAD2 was measured in the hippocampus, frontal cortex and striatum (A). The data is shown as means ± SEM for separate groups of rats (n = 5-6 per group). The immunoblot pattern of PAD2 is shown in the hippocampus after treatments with saline, Aβ alone, or Aβ and memantine (B). A quantitative analysis was carried out in the rat hippocampus (C) as arbitrary units/band intensity, and the values were expressed as a percentage of the control (saline treatment) with means ± SEM (n = 5-6). *$P < 0.05$; ***$P < 0.001$ (Bonferroni test).
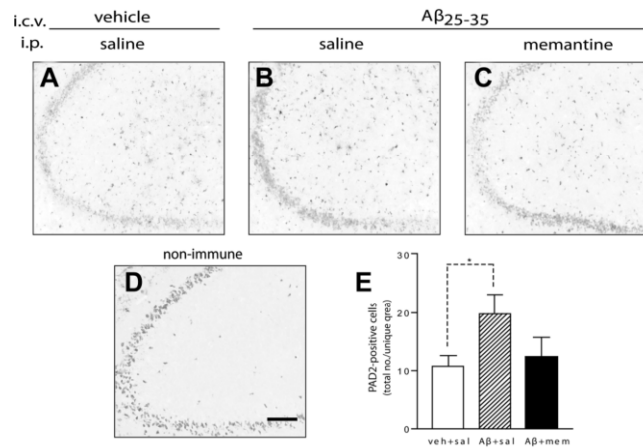
Fig. 2. The effect of memantine on Aβ-induced changes in PAD2 immunoreactivity in the rat hippocampus. The expression of PAD2 was detected using a specific PAD2 antibody in the rat hippocampus following the treatments (A-C). A non-immune IgG-probed section (D) and a quantization of PAD2-positive immunoreactivity (E) are shown. The data is shown as the means ± SEM for separate groups of rats (n = 3-4). *$P < 0.05$ (Bonferroni test). Scale bar, 100 mm (10x magnification). All the experiments (n = 3-4) were repeated twice with similar results.
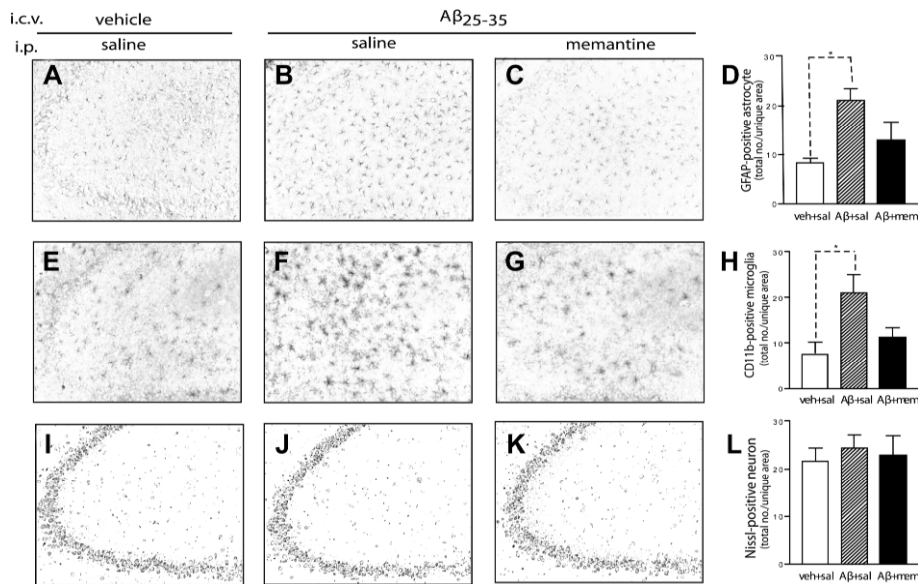

Fig. 3. The effect of repeated intracerebroventricular infusions of soluble Aβ on astrocytes, microglia and neurons. The immunohistochemical analyses of GFAP (A-D) and CD11b (E-H), the respective markers for astrocytes and microglia, were carried out in the hippocampus following treatments. The neuronal density was evaluated by Nissl staining (I-L). The data is shown as the means ± SEM for separate groups of rats (n = 3-4). **$P < 0.01$; *$P < 0.05$ (Bonferroni test). Scale bar, 100 μm (10x magnification).

The effects of Aβ infusion on GFAP and CD11b immunohistochemistry
We and other groups previously demonstrated that pre-treatment with memantine reversed the increase in astrocytes and microglia in AD model rat brain regions [9, 12]. To further assess this phenomenon, we performed an immunohistochemical analysis on the expression of astrocytes and microglia by assessing the levels of their respective marker proteins, glial fibrillary acidic protein (GFAP) and CD11b, after saline, repeated Aβ infusion or Aβ plus memantine treatments. Memantine co-treatment suppressed the activation of GFAP-postive astrocytes (Fig. 3A-D) and CD11b-positive microglia (Fig. 3E-H). To investigate whether the activation of PAD2 and of the glial marker proteins is associated with neuronal loss, we carried out Nissl staining in the hippocampus; however, we could not detect any change in cell density or number in any of the treatment groups (Fig. 3I-L).

Double immunohistochemistry
Finally, to determine the possible correlation between PAD2 and glial marker proteins, we double stained the Aβ-infused hippocampal section with anti-PAD2 coupled with the anti-GFAP or anti-CD11b antibody. Double immunofluorescence staining revealed that PAD2-positive cells coincided predominantly with GFAP-positive astrocytes (Fig. 4A-C) and a few CD11b-positive microglia (Fig. 4D-F), suggesting that the activation of PAD2expression is due to the activation of both astrocytes and microglia in the Aβ-infused rat hippocampus. Furthermore, we
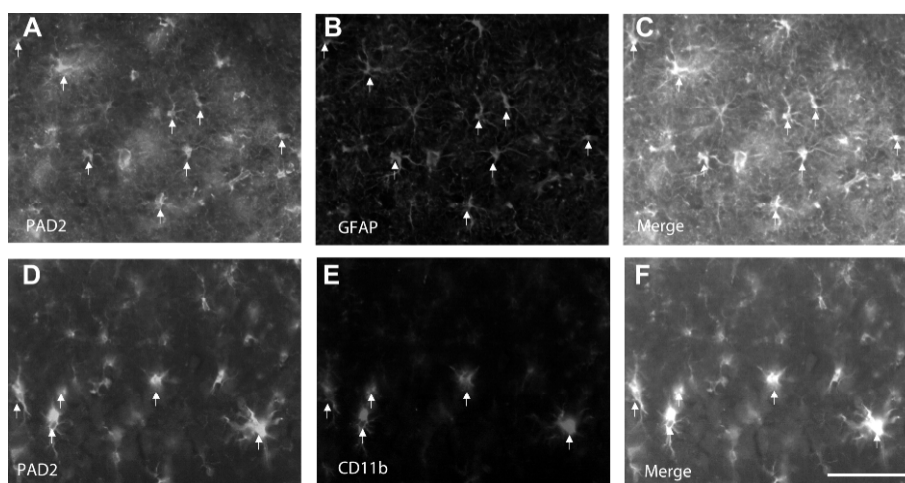

Fig. 4. Double immunostaining in the rat hippocampus after Aβ treatment. Co-localizations of GFAP (A) and PAD2 (B) (upper panel), and of CD11b (D) and PAD2 (E) (lower panel) were assessed in the hippocampus region (n = 3) after treatment with Aβ. The arrows in A and B, and D and E indicate the coincident immunoreactivity in merges C and F, respectively. Scale bar, 50 μm (40x magnification).

stained the hippocampus section with deiminated protein IgG coupled with GFAP, and found that most but not all of the GFAP-positive astrocytes co-localized with deiminated-positive immunoreactivity (Fig. 5A-C).
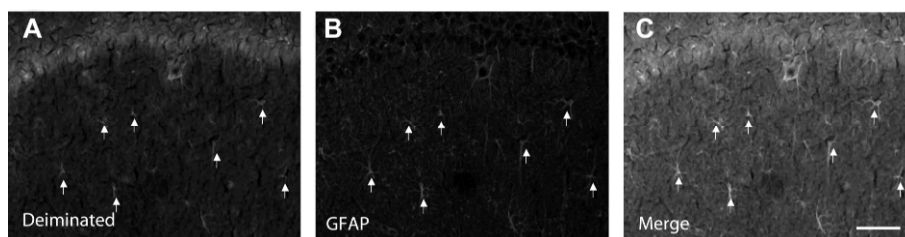

Fig. 5. Deiminated protein staining in the rat hippocampus after Aβ treatment. Co-localization of citrulline-positive (A) and GFAP-positive (B) immunoreactivity was assessed in the hippocampus (n = 3) after treatment with Aβ. The arrows in A and B indicate the coincident immunoreactivity in the merge C. Scale bar, 50 μm (20x magnification).

DISCUSSION

Here we report for the first time that a repeated intracerebroventricular infusion of soluble $Aβ_{25-35}$ elevated both the activity and protein level of PAD2 in the rat hippocampus, and that co-treatment with memantine suppressed the Aβ-induced PAD2 enzyme activation. In line with our results, increased PAD2 has been detected in hippocampal extracts from patients with AD [13].

The question arises as to how the PAD2 is activated after treatment with Aβ. One possible explanation may be the excessive accumulation of glutamate due to the inhibition of glutamate uptake in neurons following Aβ treatment [20], which then causes the activation of NMDA receptor-induced Ca^{2+} influx into the cell, as Aβ has been shown to elevate cytosolic Ca^{2+} levels in neurons *in vitro* [11]. Since PAD2 is activated in a Ca^{2+}-dependent manner, Aβ-induced disruption of Ca^{2+} homeostasis might lead to the activation of PAD2 in the AD model rat hippocampus. Another possible explanation could be the activation of glial cells following Aβ treatment, as PAD2 is localized in both astrocytes and microglia in the CNS [6, 7]. Indeed, our double immunofluorescence data clearly suggests that PAD2 is co-localized not only with activated astrocytes, but also with a few reactive microglia in our experimental paradigm. Since astrocytes play an important role in clearing the excessive glutamate released following Aβ treatment [21] and in regulating glia-neuron interaction [22], the activation of PAD2 in the astrocytes could play a beneficial role in the response to Aβ-induced toxicity. On the other hand, Aβ activation of microglia was reported to be a relatively early pathogeneic event [23] with limited Aβ clearance activity by phagocytosis [24], suggesting a protective role of PAD2 in the microglia as well. However, there are some reports that microglia

activated by Aβ can damage/kill neurons via the release of neurotoxic molecules such as inflammatory triggers, nitric oxide and reactive oxygen species [24].

To evaluate whether the increased level of PAD2 is due to the Aβ-induced neuronal dysfunction, we assessed the neuronal number and density by Nissl staining, but could not detect any noticeable change among the treatment groups. This data clearly contradicts that of Asaga [6, 7], who reported PAD2 immunoreactivity in the hyperblastic astroglia at 24 h and in the microglia at 7 days following kainate-induced neurodegeneration as measured by the disappearance of MAP2 immunoreactivity in the rat cerebrum. Similar cell death has been reported in animal experiments with fibrillar Aβ [25]. The exact reason for this disagreement is not clear, but is possibly related to the differential cellular response to kainate, the soluble and fibrillar form of Aβ. Fibrillar Aβ promotes apoptosis-mediated cell loss, whereas soluble oligomers have been implicated in the functional dysfunction of synapses other than cell loss [8, 26]. Therefore, our data suggests that treatment with soluble Aβ increased the PAD2 expression in the early stage of AD, and this event is preceded by neuronal compromise.

The increased content of PAD2 in the hippocampus is not a sufficient indication that this PAD2 is active unless the levels of its product increases in the same tissue, even though elevated PAD2-like activity is demonstrated (Fig. 1). Therefore, we examined the expression of citrullinated protein and its co-localization with GFAP. In line with previous findings, GFAP-positive citrullinated proteins were detected in the Aβ-treated rat hippocampus (Fig. 5), whereas a barely detectable signal was observed in the control (data not shown) although the control hippocampus was enriched in PAD2. From these findings, it is reasonable to assume that PAD2 normally remains inactive but becomes active, abundant and functional, and citrullinates cellular proteins when the intracellular calcium balance is upset after Aβ treatment, thus providing a mechanism for the deimination of GFAP, as seen in this study. Indeed, citrullinated-GFAP along with vimentin and myelin basic protein (MBP) has been reported in the hippocampal extracts from patients with AD [13]. Astrocytes act as a reservoir of arginine storage in the brain and thus appear as a common target of PAD2 activation in pathological conditions. It is still not known whether the citrullination of GFAP is protective for surviving astrocytes resulting in the proliferation of the cells. However, Louw et al. [27] showed reduced PAD2 activity in the brain homogenate following Aβ treatment, which is in opposition to our results and those of other researchers. The apparent discrepancy may be due to the diverse Aβ species used in these studies. Although we used soluble Aβ, similarly to the Louw study, we considered that most of the soluble Aβ is converted into oligomers in our experimental time frame, and it is well established that the oligomeric rather than the monomeric form of Aβ is responsible for memory impairment and LTP inhibition [28]. Therefore, we assumed that the earlier pathological response that we demonstrated in this study is due to the soluble oligomeric form of Aβ.

Interestingly, co-treatment with memantine partially suppressed the increase in PAD2 in the Aβ-treated rat brain; however, the molecular mechanism is not well understood. Earlier studies have shown that memantine can restore LTP impairment induced by NMDA receptor hyperactivity, and can also reverse cognitive behavioral deficits in animal models of AD [29]. In addition, memantine decreases the basal level of intracellular Ca^{2+} in HEK 293 cells [30]. Treatment with memantine might have the following consequences: memantine blocks the NMDA receptor, resulting in a decrease in extra-cellular glutamate and intracellular Ca^{2+} concentrations [25] and suppressing the astrocyte and microglial activation [9], thereby attenuating PAD2 activation.

In conclusion, we used combined biochemical and immunohistochemical approaches to show the effect of Aβ on PAD2 expression. Our results suggest that repeated infusion with soluble Aβ increased the level of PAD2 in association with astrocytes and microglia in the AD model rat hippocampus and might play a role in the pathogenesis of AD. Co-treatment with memantine partially suppressed the glial cell-derived PAD2 increment. Since PADs catalyze the conversion of protein arginine to protein citrulline residue, which is considered a potential marker for neurodegeneration [6, 7, 10], further studies are needed to evaluate the role of protein deimination in the AD animal model.

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