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Fe^{3+} Facilitates Endocytic Internalization of Extracellular $A\beta_{1–42}$ and Enhances $A\beta_{1–42}$-Induced Caspase-3/Caspase-4 Activation and Neuronal Cell Death

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Abstract

Amyloid β (Aβ) peptide is a critical causative factor in Alzheimer's disease (AD) and of a variety of fragmented Aβ peptides $A\beta_{1–42}$ thought to exhibit the most neurotoxic effect. The present study investigated the effects of Fe^{3+} on $A\beta_{1–42}$ internalization and $A\beta_{1–42}$-induced caspase activation and neurotoxicity using mouse hippocampal slices and cultured PC-12 cells. Extracellularly applied $A\beta_{1–42}$ increased the cell-associated $A\beta_{1–42}$ levels in a concentration-dependent manner, and the effect was enhanced by adding Fe^{3+}. Fe^{3+}-induced enhancement of the cell-associated $A\beta_{1–42}$ levels was significantly inhibited by the endocytosis inhibitors dynasore and methyl-β-cyclodextrin. $A\beta_{1–42}$ reduced PC-12 cell viability in a concentration-dependent manner, and further reduction of the cell viability was obtained with Fe^{3+}. $A\beta_{1–42}$-induced reduction of cell viability was not affected by A187, an antagonist of amylin-3 receptor. $A\beta_{1–42}$ activated caspase-3, caspase-4, and caspase-8 to a variety of degrees and Fe^{3+} further enhanced $A\beta_{1–42}$-induced activation of caspase-3 and caspase-4. Taken together, these results indicate that Fe^{3+} accelerates endocytic internalization of extracellular $A\beta_{1–42}$, enhances $A\beta_{1–42}$-induced caspase-3/caspase-4 activation, and promotes $A\beta_{1–42}$-induced neuronal cell death, regardless of amylin receptor.

Keywords Amyloid $\beta_{1–42}$ · Fe^{3+} · Internalization · Endocytosis · Neuronal cell death · Caspase activation

Introduction

Alzheimer's disease (AD) is a neurodegenerative disease with severe dementia, which is characterized by extracellular amyloid plaques, intraneuronal tangles, and cerebrovascular amyloid deposits. The extracellular plaques contain amyloid β (Aβ) peptides such as $A\beta_{1–40}$ and $A\beta_{1–42}$, which are derived by two proteolytic cleavages from the larger amyloid precursor protein (APP). A 99-residue C-terminal fragment (C99) of APP is generated by β-secretase cleavage (BACE1), followed by γ-secretase-mediated procession to generate Aβ peptides [1]. The extracellular amyloid plaques are predominantly formed in the hippocampus and cerebral cortex linked to cognitive function, while the cerebrovascular amyloid deposits are formed in cerebral arteries and capillaries, causing cerebral amyloid angiopathy (CAA). About 80% of AD patients were shown to have CAA [2].

Whereas Aβ has long been pointed as a root cause of AD pathology, how Aβ induces AD is as yet under discussion. In addition, it remains to be elucidated which of the extracellular Aβ deposition or intracellular Aβ accumulation initiates the AD process. Intriguingly, intraneuronal Aβ accumulation precedes the accumulation in the extracellular space [3]. Intraneuronal $A\beta_{1–42}$ accumulation is mainly found in the entorhinal cortex and the hippocampus consistent with the regions of amyloid plaque accumulation in the human AD brain [4, 5]. Intraneuronal $A\beta_{1–42}$ accumulation is also detected in the brain of AD model mice with presenilin-1 mutation, where there is no amyloid plaque [6]. Aβ is recognized to impair neuronal functions in association with apoptosis and oxidative injury still prior to formation of amyloid plaque and neurofibrillary tangles [7, 8]. Overall, intraneuronal Aβ accumulation may become a focus for extracellular plaque formation following neuronal death and lysis [9] and therefore, it may be the first step to cause neurodegeneration in AD.

It has been shown that there are significant differences in the intracellular uptake and catabolism of Aβ, depending upon Aβ length [10]; uptake of $A\beta_{1–42}$ into differentiated PC12

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cells is greater than that of $A\beta_{1–40}$ and $A\beta_{1–28}$, and internalized $A\beta_{1–42}$ remains for at least 3 days, while internalized $A\beta_{1–40}$ and $A\beta_{1–28}$ are eliminated with a half-life of 1 h. Interestingly, the $A\beta_{1–42}$ uptake is inhibited by neuraminidase. This raises the possibility that $A\beta_{1–42}$ is taken up into cells due to sialylated receptor-mediated endocytosis. Uptake of $A\beta_{1–40}$ and $A\beta_{1–42}$ into cells is also obtained with human neuroblastoma cells [11]. Intraneuronal $A\beta_{1–42}$ accumulation is facilitated by α7 nicotinic acetylcholine receptor [12] or NMDA receptor [13], suggesting the implication of these receptors in the $A\beta_{1–42}$ uptake into neurons.

Extracellular Aβ aggregation is thought to be one of the key pathogenic events in AD. The metal ions such as Al^{3+}, Zn^{2+}, Cu^{2+}, and Fe^{2+} are required for Aβ aggregation [14–19]. NF-κB-mediated upregulation of the amyloid precursor protein (APP) gene occurs prior to amyloid plaque formation in response to Al^{3+} or Fe^{3+} [15]. Fe^{2+} promotes $A\beta_{1–42}$ oligomerization by enhancing the peptide-peptide interaction [16]. Abnormally high concentrations of Zn^{2+}, Cu^{2+}, and Fe^{3+}, but not Fe^{2+}, are present along with Aβ in the amyloid plaques in AD [17–19]. During Aβ aggregation, Zn^{2+} exclusively promotes the annular protofibril formation without undergoing a nucleation process, whereas Cu^{2+} and Fe^{3+} inhibit fibril formation by prolonging the nucleation phases [20]. Iron may promote Aβ toxicity in AD by delaying well-ordered aggregates of Aβ [21]. Iron, alternatively, may act as a specific cofactor for distinct oxidation- and aggregation-dependent Aβ toxicity mechanisms [22]. Upregulation of the stress-related genes is observed in the AD brain, and the combination of iron and aluminum together upregulates expression of the genes synergistically [23]. The micro-RNAs (miRNAs) miRNA-125b and miRNA-146a, alternatively, are upregulated in the AD brain, and the combination of iron plus aluminum sulfate upregulates expression of NF-κB-sensitive miRNA-125b and miRNA-146a synergistically [24]. These findings explain that Al^{3+} and Fe^{3+} play a critical role in AD pathogenesis. Moreover, emerging evidence has pointed to co-localization of iron and Aβ, possibly responsible for mild cognitive impairment (MCI) and AD [25–29].

Little is known about the effects of Fe^{3+} alone on intracellular $A\beta_{1–42}$ uptake and $A\beta_{1–42}$-induced caspase activation and neurotoxicity. The present study aimed at understanding these questions. The results show that Fe^{3+} accelerates endocytic internalization of extracellular $A\beta_{1–42}$ into cells and enhances $A\beta_{1–42}$-induced caspase-3/caspase-4 activation and neuronal cell death.

Methods

Animal Care

All procedures have been approved by the Animal Care and Use Committee at Hyogo College of Medicine, where the corresponding author Nishizaki worked as a professor previously, and were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Cell Culture

PC-12 cells, obtained from RIKEN Cell Bank (Tsukuba, Japan), were cultured in DMEM with 10% (*v*/*v*) heat-inactivated FBS and 10% (*v*/*v*) heat-inactivated horse serum supplemented with penicillin (100 U/ml) and streptomycin (0.1 mg/mL) in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. PC-12 cells were differentiated by treatment with nerve growth factor (100 ng/mL) for 6 days.

$A\beta_{1–42}$ Internalization

$A\beta_{1–42}$ (Abcam, Cambridge, UK) were dissolved in dimethyl sulfoxide (DMSO) and diluted to the concentration desired with an external solution.

C57BL/6J mice (male, 8 weeks of age) were purchased from Japan SLC Inc. (Shizuoka, Japan), and eight mice were used for the present study. After decapitation under ether anesthesia, the hippocampus was isolated from the mouse brain and hippocampal slices (400 μm in thickness) were prepared using a microtome. Mouse hippocampal slices were treated with $A\beta_{1–42}$ for 1–3 h in a standard artificial cerebrospinal fluid (117 mM NaCl, 3.6 mM KCl, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, and 11.5 mM glucose) oxygenated with 95% O_2 and 5% CO_2 at 34 °C. Then, slices were homogenized by sonication in TBS-T [150 mM NaCl, 0.1% (*v*/*v*) Tween-20 and 20 mM Tris, pH 7.5] containing 1% (*v*/*v*) protease inhibitor cocktail after enough washing.

PC-12 cells were treated with DMSO or $A\beta_{1–42}$ (Abcam) in the culture medium containing with and without $FeCl_3$. Dynasore or methyl-β-cyclodextrin was applied to cells 1 h prior to treatment with DMSO or $A\beta_{1–42}$. Then, cells were homogenized by sonication in TBS-T containing 1% (*v*/*v*) phosphatase inhibitor cocktail after enough washing.

Homogenates from mouse hippocampal slices and PC-12 cells were dissolved in 30 μL of a sodium dodecyl sulfate (SDS) sample buffer [0.2 mM Tris, 0.05% (*w*/*v*) SDS, and 20% (*v*/*v*) glycerol, pH 6.8]. Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes. Blotting membranes were blocked with TBS-T containing 5% (*w*/*v*) bovine serum albumin and in turn, incubated with antibodies against $A\beta_{1–42}$ antibody (Abcam) and β-actin (Cell Signaling, Beverly, MA, USA). After washing, membranes were reacted with a horseradish peroxidase-conjugated goat anti-mouse IgG antibody. Immunoreactivity was detected with an ECL kit (GE Healthcare) and visualized using a chemiluminescence

detection system (GE Healthcare). Protein concentrations for each sample were determined with a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA).

Intracellular Localization of $A\beta_{1-42}$

After 72-h treatment with $A\beta_{1-42}$ (Abcam), PC-12 cells were fixed with 4% (*w*/*v*) paraformaldehyde, permeabilized with 0.3% (*v*/*v*) Triton X-100, and blocked with 10% (*v*/*v*) goat serum at room temperature. The fixed cells were reacted with an anti-$A\beta_{1-42}$ antibody (Abcam), which is reactive with $A\beta_{42}$ and does not cross-react with $A\beta_{1-40}$, full-length APP, sAPPβ, or sAPPα, at 4 °C overnight, followed by a goat anti-mouse IgG antibody conjugated with Alexa 633 (Molecular Probes, Eugene, OR, USA) for 60 min at room temperature. The nucleus was stained with 4′,6-diamidino-2-phenylindole (DAPI). Cell imaging was observed under a laser scanning confocal microscope (LSM 510, Carl Zeiss Co., Ltd., Oberkochen, Germany).

Cell Viability

PC-12 cell viability was evaluated by a dye staining method using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (DOJINDO, Kumamoto, Japan) by the method as previously described [30].

Enzymatic Assay of Caspase-3, Caspase-4, Caspase-8, and Caspase-9 Activities

Caspase activity was measured using a caspase fluorometric assay kit (Ac-Asp-Glu-Val-Asp-MCA for a caspase-3 substrate peptide; Ac-Leu-Glu-Val-Asp-AFC for a caspase-4 substrate peptide; Ac-Ile-Glu-Thr-Asp-MCA for a caspase-8 substrate peptide; and Ac-Leu-Glu-His-Asp-MCA for a caspase-9 substrate peptide) by the method as previously described [31]. Briefly, PC-12 cells were harvested before and after treatment with $A\beta_{1-42}$ (Abcam) in the presence and absence of $FeCl_3$ and then centrifuged at 1200 rpm for 5 min at 4 °C. The pellet was incubated on ice in a cell lysis buffer for 10 min and reacted with the fluorescently labeled tetrapeptide at 37 °C for 2 h. The fluorescence was measured at an excitation wavelength of 380 nm and an emission wavelength of 460 nm for caspase-3, caspase-8, and caspase-9 or an excitation wavelength of 400 nm and an emission wavelength of 505 nm for caspase-4 with a fluorometer (Fluorescence Spectrometer, F-4500, HITACHI, Japan).

Statistical Analysis

Statistical analysis was carried out using analysis of variance (ANOVA) followed by a Bonferroni correction.

Results

Extracellular $A\beta_{1-42}$ Is Internalized into Cells

My initial attempt was to see whether extracellular $A\beta_{1-42}$ is internalized into cells. When mouse hippocampal slices were treated with $A\beta_{1-42}$ for 1 or 3 h, the cell-associated $A\beta_{1-42}$ levels in hippocampal cells rose in an extracellular $A\beta_{1-42}$ concentration (1–10 μM)-dependent manner, reaching nearly 40-fold of the basal levels at 10 μM (Fig. 1a, b). This indicates that extracellular $A\beta_{1-42}$ is internalized into cells.

To obtain further evidence for $A\beta_{1-42}$ internalization, the immuno-fluorescence cytochemistry was carried out using PC-12 cells. The fluorescent signals for $A\beta_{1-42}$ were detected around the nucleus in cells treated with $A\beta_{1-42}$ for 72 h (Fig. 2b), while no signal was found in cells without $A\beta_{1-42}$ treatment (Fig. 2a). This indicates that extracellular $A\beta_{1-42}$ is actually taken up into cells.

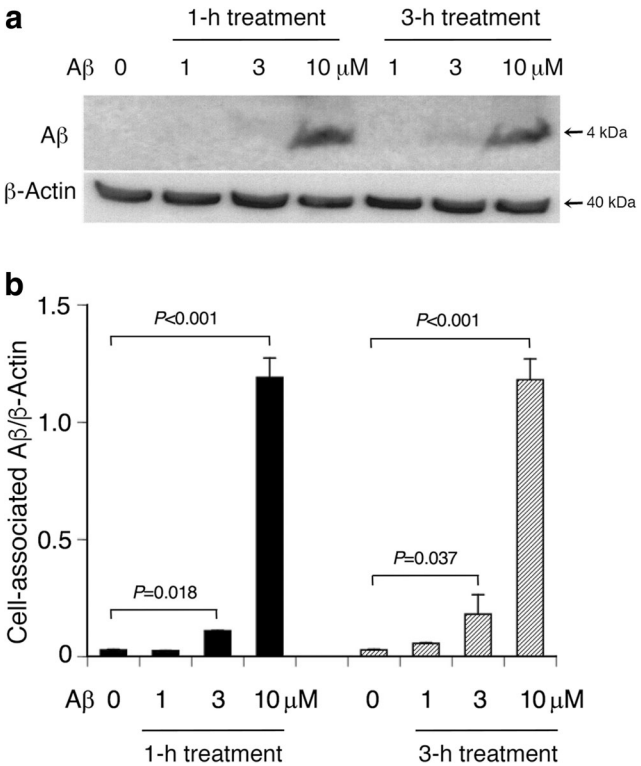

Fig. 1 Extracellular $A\beta_{1-42}$ is internalized into cells. Mouse hippocampal slices were treated with $A\beta_{1-42}$ (Aβ) at concentrations as indicated for 1 or 3 h and lysed, followed by Western blotting in the lysates using an anti-$A\beta_{1-42}$ antibody. Typical Western blot image is shown in (**a**). **b** In the graph, each column represents the mean (± SEM) signal intensity for $A\beta_{1-42}$ monomer normalized by the signal intensity for β-actin ($n = 4$ independent Western blot results). *P* values, ANOVA followed by a Bonferroni correction

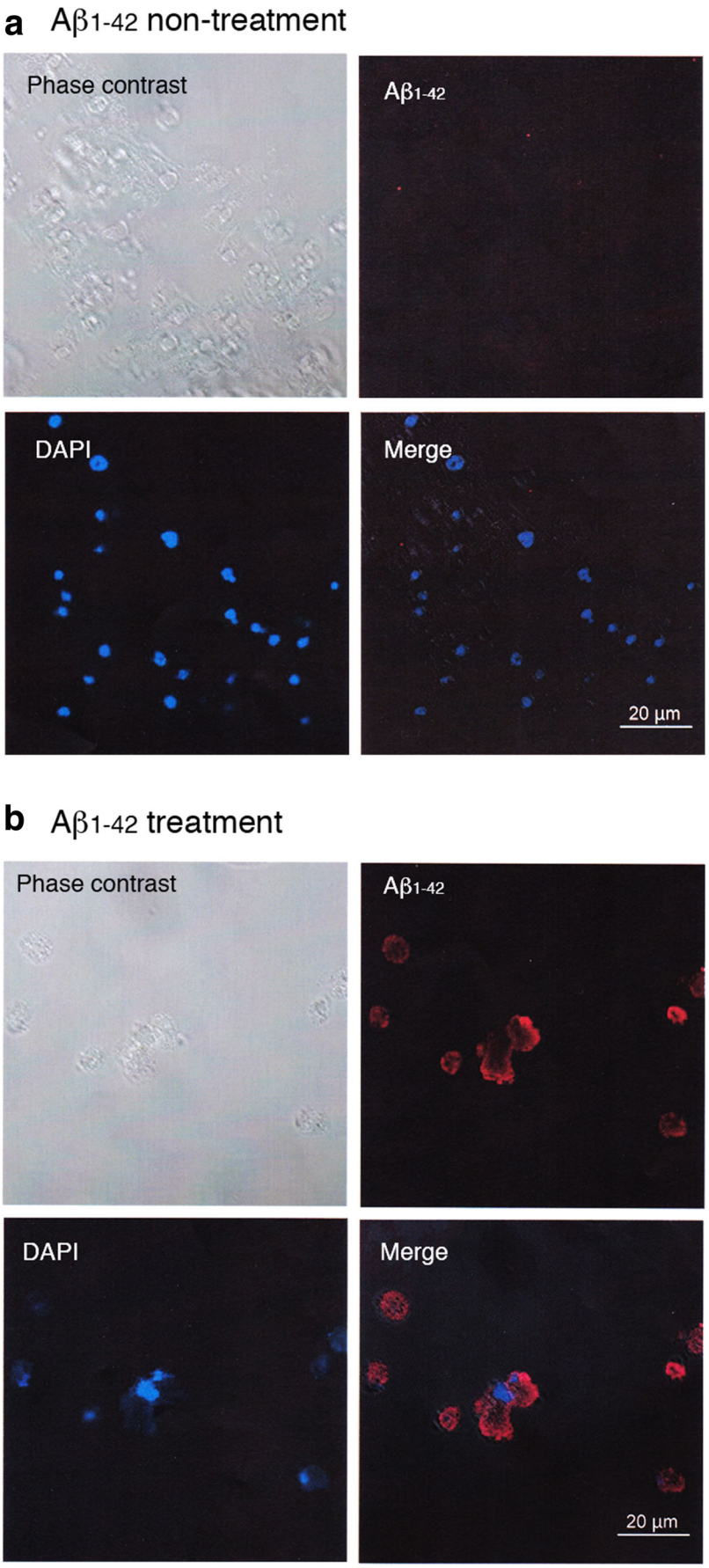

Fig. 2 Intracellular localization of $A\beta_{1-42}$. PC-12 cells were untreated (**a**) and treated with $A\beta_{1-42}$ (30 μM) for 72 h (**b**), and then immuno-fluorescence cytochemistry was carried out. $A\beta_{1-42}$, red color; nucleus, blue color

Fe^{3+} Accelerates Endocytic Internalization of Extracellular $A\beta_{1–42}$ into Cells

Extracellularly applied $A\beta_{1–42}$ significantly raised the cell-associated $A\beta_{1–42}$ levels in PC-12 cells (Fig. 3). Fe^{3+} alone had no effect on the cell-associated $A\beta_{1–42}$ levels, but a huge increase in the cell-associated $A\beta_{1–42}$ levels was obtained by co-treatment with Fe^{3+} and $A\beta_{1–42}$ (Fig. 3). The effect of Fe^{3+} was significantly inhibited by the endocytosis inhibitors dynasore and methyl-β-cyclodextrin (Fig. 3). Taken together, these results indicate that Fe^{3+} facilitates endocytic internalization of extracellular $A\beta_{1–42}$.

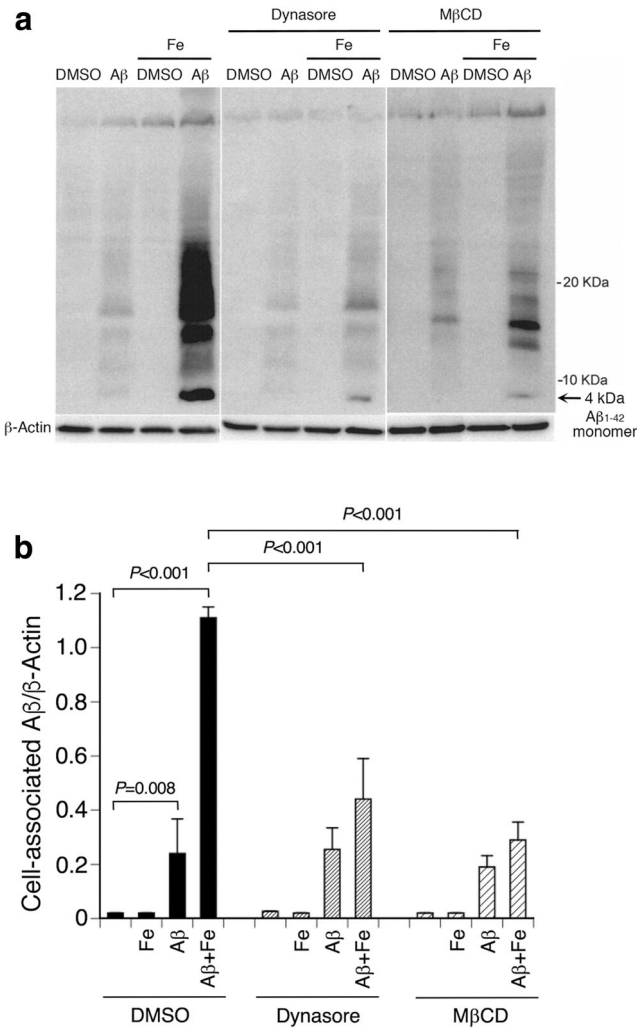

Fig. 3 Fe^{3+} accelerates endocytic internalization of extracellular $A\beta_{1–42}$. PC-12 cells were treated with DMSO or $A\beta_{1–42}$ (Aβ) (3 μM) in the presence and absence of $FeCl_3$ (Fe) (1 mM) for 24 h following 1-h pretreatment dynasore (80 μM) or methyl-β-cyclodextrin (MβCD) (1 mM) and lysed, followed by Western blotting in the lysates using an anti-$A\beta_{1–42}$-antibody. Typical Western blot image is shown in (**a**). Arrow indicates $A\beta_{1–42}$ monomer at 4 kDa. **b** In the graph, each column represents the mean (± SEM) signal intensity for $A\beta_{1–42}$ monomer normalized by the signal intensity for β-actin ($n = 4$ independent Western blot results). *P* values, ANOVA followed by a Bonferroni correction

Fe^{3+} Promotes $A\beta_{1–42}$-Induced Neuronal Cell Death

In the MTT assay, $A\beta_{1–42}$ reduced PC-12 cell viability in a concentration (1–5 μM)-dependent manner (Fig. 4). This implies that $A\beta_{1–42}$ induces neuronal cell death.

Co-treatment with Fe^{3+} and $A\beta_{1–42}$ significantly enhanced the effect of $A\beta_{1–42}$ on cell viability, although Fe^{3+} alone had no significant effect on cell viability (Fig. 4). This indicates that Fe^{3+} promotes $A\beta_{1–42}$-induced neuronal cell death.

$A\beta_{1–42}$-induced reduction of PC-12 cell viability was not affected by AC187, an antagonist of amylin-3 receptor (Fig. 5). This indicates that $A\beta_{1–42}$ induces neuronal cell death, regardless of amylin receptor.

Fe^{3+} Enhances $A\beta_{1–42}$-Induced Activation of Caspase-3/Caspase-4

Treatment with Fe^{3+} alone or $A\beta_{1–42}$ alone for 24 h did not affect the activities of caspase-3, caspase-4, caspase-8, and caspase-9 in PC-12 cells, but co-treatment with Fe^{3+} and $A\beta_{1–42}$ enhanced the activities of caspase-3 and caspase-4 (Fig. 6a). Treatment with Fe^{3+} alone for 48 h had no significant effect on the activities of caspase-3, caspase-4, caspase-8, and caspase-9 (Fig. 6b). In contrast, treatment with $A\beta_{1–42}$ alone for 48 h significantly enhanced the activities of caspase-3, caspase-4, and caspase-8, and further enhancement in the activities of caspase-3 was obtained by adding Fe^{3+} (Fig. 6b). Overall, these results suggest that Fe^{3+} enhances $A\beta_{1–42}$-induced activation of caspase-3/caspase-4, thereby promoting $A\beta_{1–42}$-induced apoptotic cell death.

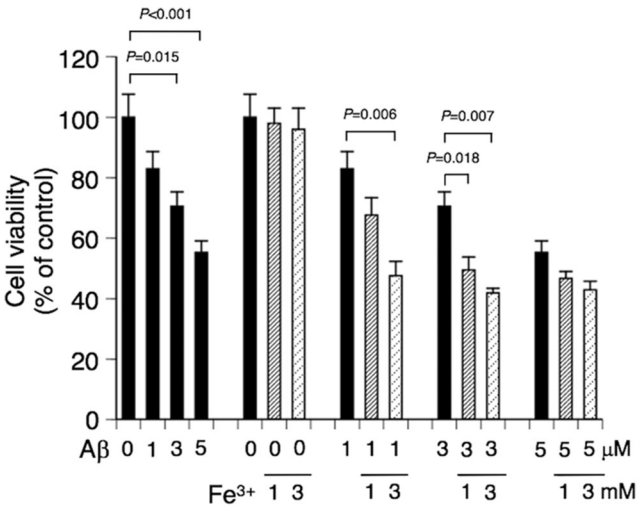

Fig. 4 Fe^{3+} promotes $A\beta_{1–42}$-induced neuronal cell death. PC-12 cells were treated with and without $A\beta_{1–42}$ (Aβ) at concentrations as indicated in the presence and absence of $FeCl_3$ (Fe^{3+}) (1 or 3 mM) for 48 h, and cell viability was quantified with an MTT assay. In the graph, each column represents the mean (± SEM) percentage of basal levels (MTT intensities in cells untreated with $A\beta_{1–42}$ in the absence of $FeCl_3$) ($n = 4$ independent MTT assays). *P* values, ANOVA followed by a Bonferroni correction

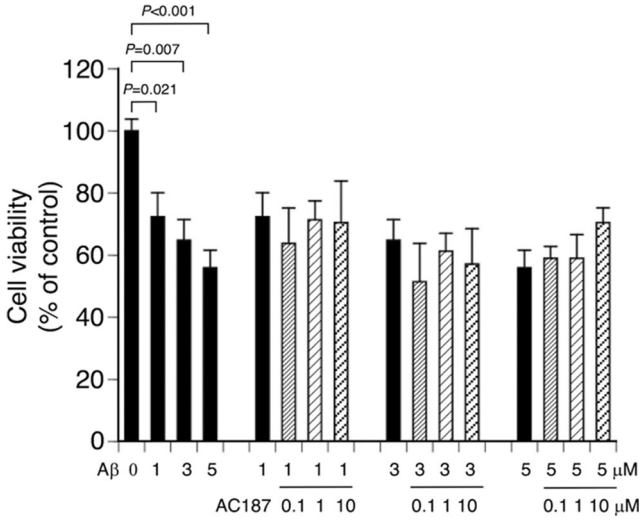

Fig. 5 $A\beta_{1–42}$ induces neuronal cell death, regardless of amylin-3 receptor. PC-12 cells were treated with $A\beta_{1–42}$ (Aβ) at concentrations as indicated in the presence and absence of AC187 at concentrations as indicated for 48 h, and cell viability was quantified with an MTT assay. In the graph, each column represents the mean (± SEM) percentage of basal levels (MTT intensities in cells untreated with $A\beta_{1–42}$ in the absence of AC187) ($n = 4$ independent MTT assays). *P* values, ANOVA followed by a Bonferroni correction

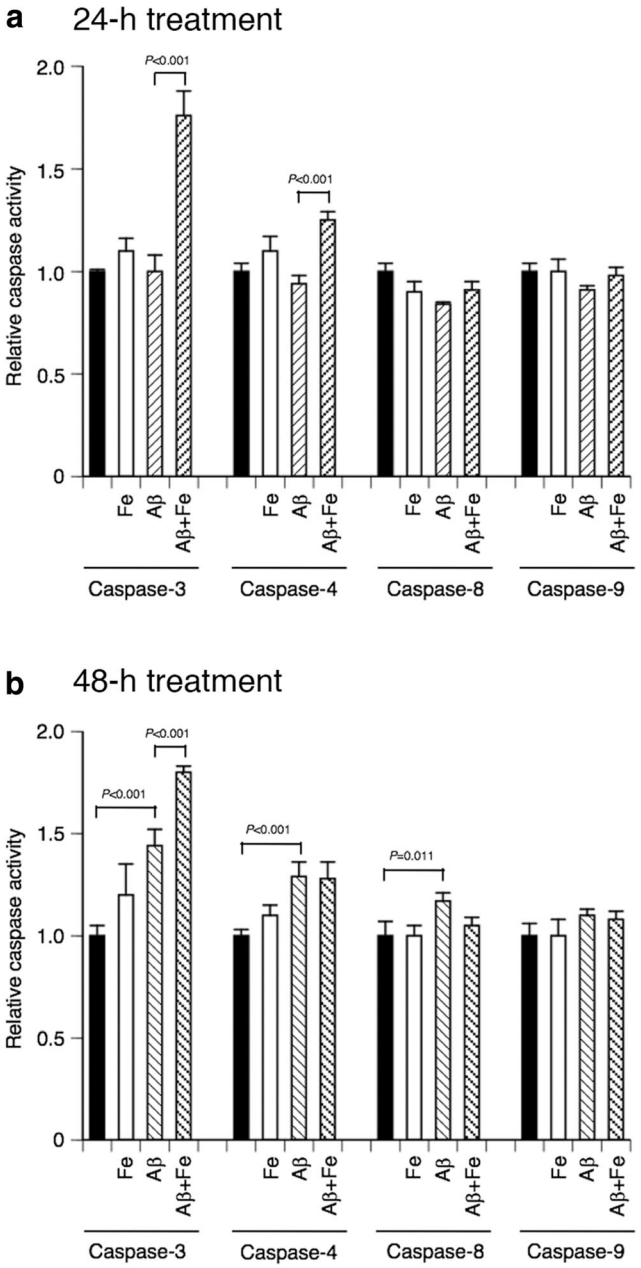

Fig. 6 Fe^{3+} enhances $A\beta_{1–42}$-induced activation of caspase-3/caspase-4. PC-12 cells were treated with and without $A\beta_{1–42}$ (Aβ) (3 μM) in the presence and absence of $FeCl_3$ (Fe) (1 mM) for 24 h (**a**) or 48 h (**b**), and activities of caspase-3, caspase-4, caspase-8, and caspase-9 were assayed. In the graphs, each column represents the mean (± SEM) ratio against basal caspase activities in cells untreated with $A\beta_{1–42}$ and $FeCl_3$ ($n = 8$ independent caspase assays). *P* values, ANOVA followed by a Bonferroni correction

Discussion

Several avenues of studies have shown that Aβ is prerequisite for pathogenesis of AD and that Aβ serves as an initiator. Extracellular Aβ, especially $A\beta_{1–42}$, is well-recognized to exhibit a neurotoxic effect. The role of intraneuronal Aβ in the AD pathogenesis, however, largely remains unclear.

In our earlier study, $A\beta_{1–42}$ was preferentially present in the plasma membrane components of brain lysates from 5xFAD mouse, an animal model of AD [32], suggesting intraneuronal accumulation of $A\beta_{1–42}$. In the present study, treatment with extracellular $A\beta_{1–42}$ for 3 h raised the cell-associated $A\beta_{1–42}$ levels in a concentration (1–10 μM)-dependent manner in mouse hippocampal slices and intracellular accumulation of $A\beta_{1–42}$ in PC-12 cells was observed after 72-h treatment with extracellular $A\beta_{1–42}$. These results provide further evidence for internalization of extracellular $A\beta_{1–42}$ into neuronal cells.

Fe ions exert a variety of effects on Aβ pathogenic characteristics, which include acceleration of extracellular Aβ aggregation and oligomerization, suppression of fibril formation, and enhancement of Aβ toxicity [16–22]. In the present study, Fe^{3+} facilitated internalization of extracellular $A\beta_{1–42}$ into cells. To my knowledge, this is the first to show the implication of Fe^{3+} in the $A\beta_{1–42}$ internalization.

One argues that misfolded Aβ is spread into cells by a prion-like mechanism [33]. How $A\beta_{1–42}$ is internalized into cells, however, is not fully understood. The facilitatory effect of Fe^{3+} on $A\beta_{1–42}$ internalization was canceled by the endocytosis inhibitors dynasore and methyl-β-cyclodextrin. This suggests that extracellular $A\beta_{1–42}$ is internalized by endocytosis and that Fe^{3+} facilitates endocytic internalization of extracellular $A\beta_{1–42}$.

In the present study, $A\beta_{1–42}$ reduced PC-12 cell viability in a concentration (1–5 μM)-dependent manner, and the addition of Fe^{3+} further enhanced $A\beta_{1–42}$-induced reduction of cell viability. This suggests that $A\beta_{1–42}$ induces neuronal cell

death and that Fe^{3+} promotes the effect of $A\beta_{1-42}$. Intracellular Aβ induces endoplasmic reticulum (ER) stress, to activate unfolded protein response (UPR), leading to activation of caspase-4 followed by the effector caspase-3, to induce apoptosis [34]. Co-treatment with Fe^{3+} and $A\beta_{1-42}$ for 24 h enhanced the activities of caspase-3 and caspase-4 in PC-12 cells, although Fe^{3+} alone or $A\beta_{1-42}$ alone had no effect. The activities of caspase-3, caspase-4, and caspase-8 were enhanced by 48-h treatment with $A\beta_{1-42}$ alone, and further enhancement in the caspase-3 activity was obtained by adding Fe^{3+}. Aβ is shown to inhibit cellular MTT reduction by stimulating MTT formazan exocytosis [35]. This implies that the cellular toxicity of Aβ cannot be estimated accurately by MTT assay. The results of MTT assay in the present study, however, together with the results of caspase assay, allow drawing a conclusion that Fe^{3+} enhances $A\beta_{1-42}$-induced caspase-3/4 activation, thereby promoting neuronal cell death.

$A\beta_{1-42}$ as well as the islet amyloid polypeptide amylin directly bind to and activate amylin receptor-3, an isoform of the amylin receptor family, involving multiple signal transduction pathways linked to activation of protein kinase A, mitogen-activated protein kinase, Akt, and cFos through an increase in cytosolic cAMP and Ca^{2+} [36]. Accumulating evidence has pointed to the implication of amylin receptor-3 in Aβ toxicity [37, 38]. Amylin receptor, therefore, is currently highlighted as a potential therapeutic target for AD [39–41]. $A\beta_{1-42}$ and human amylin are shown to upregulate expression of caspase-3, caspase-6, caspase-9, BID, and XIAP, to induce apoptosis in human fetal neurons [38]. Unexpectedly, $A\beta_{1-42}$-induced reduction of PC-12 cell viability here was not affected by the antagonist of amylin-3 receptor AC187. This accounts for $A\beta_{1-42}$-induced apoptosis through a pathway independent of amylin-3 receptor. Internalized $A\beta_{1-42}$, therefore, might activate caspase-3, caspase-4, and caspase-8 directly or indirectly, responsible for induction of apoptotic cell death. To address this question, further experiments need to be carried out.

In summary, the results of the present study show that Fe^{3+} facilitates endocytic internalization of extracellular $A\beta_{1-42}$, enhances $A\beta_{1-42}$-induced activation of caspase-3/caspase-4, and promotes $A\beta_{1-42}$-induced neuronal cell death. This may provide fresh insight into the role of Fe^{3+} in $A\beta_{1-42}$ toxicity linked to AD pathology.

Compliance with Ethical Standards

Conflict of Interest The author declares that he has no conflict of interest.

References

1. Tomita T (2017) Aberrant proteolytic processing and therapeutic strategies in Alzheimer disease. Adv Biol Regul 64:33–38
2. Attems J, Quass M, Jellinger KA, Lintner F (2007) Topographical distribution of cerebral amyloid angiopathy and its effect on cognitive decline are influenced by Alzheimer disease pathology. J Neurol Sci 257(1–2):49–55
3. Wirths O, Multhaup G, Bayer TA (2004) A modified β-amyloid hypothesis: intraneuronal accumulation of the β-amyloid peptide - the first step of a fatal cascade. J Neurochem 91(3):513–520
4. Mochizuki A, Tamaoka A, Shimohata A, Komatsuzaki Y, Shoji S (2000) Aβ42-positive non-pyramidal neurons around amyloid plaques in Alzheimer's disease. Lancet 355(9197):42–43
5. Gouras GK, Tsai J, Naslund J, Vincent B, Edgar M, Checler F, Greenfield JP, Haroutunian V et al (2000) Intraneuronal Aβ42 accumulation in human brain. Am J Pathol 156(1):15–20
6. Chui DH, Tanahashi H, Ozawa K, Ikeda S, Checler F, Ueda O, Suzuki H, Araki W et al (1999) Transgenic mice with Alzheimer presenilin 1 mutations show accelerated neurodegeneration without amyloid plaque formation. Nat Med 5(5):560–564
7. LaFerla FM, Troncoso JC, Strickland DK, Kawas CH, Jay G (1997) Neuronal cell death in Alzheimer's disease correlates with apoE uptake and intracellular Aβ stabilization. J Clin Invest 100(2):310–320
8. Guo Q, Fu W, Xie J, Luo H, Sells SF, Geddes JW, Bondada V, Rangnekar VM et al (1998) Par-4 is a mediator of neuronal degeneration associated with the pathogenesis of Alzheimer disease. Nat Med 4(8):957–962
9. D'Andrea MR, Nagele RG, Wang HY, Peterson PA, Lee DH (2001) Evidence that neurones accumulating amyloid can undergo lysis to form amyloid plaques in Alzheimer's disease. Histopathology 38(2):120–134
10. Burdick D, Kosmoski J, Knauer MF, Glabe CG (1997) Preferential adsorption, internalization and resistance to degradation of the major isoform of the Alzheimer's amyloid peptide, Aβ1-42, in differentiated PC12 cells. Brain Res 746(1–2):275–284
11. Morelli L, Prat MI, Castano EM (1999) Differential accumulation of soluble amyloid β peptides 1-40 and 1-42 in human monocytic and neuroblastoma cell lines. Implications for cerebral amyloidogenesis. Cell Tissue Res 298(2):225–232
12. Nagele RG, D'Andrea MR, Anderson WJ, Wang HY (2002) Intracellular accumulation of β-amyloid1–42 in neurons is facilitated by the α7 nicotinic acetylcholine receptor in Alzheimer's disease. Neuroscience 110(2):199–211
13. Jellinger KA (2002) Alzheimer disease and cerebrovascular pathology: an update. J Neural Transm 109(5–6):813–836
14. Mantyh PW, Ghilardi JR, Rogers S, DeMaster E, Allen CJ, Stimson ER, Maggio JE (1993) Aluminum, iron, and zinc ions promote aggregation of physiological concentrations of beta-amyloid peptide. J Neurochem 61(3):1171–1174
15. Walton JR, Wang MX (2009) APP expression, distribution and accumulation are altered by aluminum in a rodent model for Alzheimer's disease. J Inorg Biochem 103(11):1548–1554
16. Boopathi S, Kolandaivel P (2016) Fe^{2+} binding on amyloid β-peptide promotes aggregation. Proteins 84(9):1257–1274
17. Lovell MA, Robertson JD, Teesdale WJ, Campbell JL, Markesbery WR (1998) Copper, iron and zinc in Alzheimer's disease senile plaques. J Neurol Sci 158(1):47–52
18. Frederickson CJ, Koh JY, Bush AI (2005) The neurobiology of zinc in health and disease. Nat Rev Neurosci 6(6):449–462
19. James SA, Churches QI, de Jonge MD, Birchall IE, Streltsov V, McColl G, Adlard PA, Hare DJ (2017) Iron, copper, and zinc concentration in Aβ plaques in the APP/PS1 mouse model of Alzheimer's disease correlates with metal levels in the surrounding neuropil. ACS Chem Neurosci 8(3):629–637
20. Chen WT, Liao YH, Yu HM, Cheng IH, Chen YR (2011) Distinct effects of Zn^{2+}, Cu^{2+}, Fe^{3+}, and Al^{3+} on amyloid-β stability, oligomerization, and aggregation: amyloid-β destabilization promotes annular protofibril formation. J Biol Chem 286(11):9646–9656

21. Liu B, Moloney A, Meehan S, Morris K, Thomas SE, Serpell LC, Hider R, Marciniak SJ et al (2011) Iron promotes the toxicity of amyloid β peptide by impeding its ordered aggregation. J Biol Chem 286(6):4248–4256
22. Ott S, Dziadulewicz N, Crowther DC (2015) Iron is a specific cofactor for distinct oxidation- and aggregation-dependent Aβ toxicity mechanisms in a Drosophila model. Dis Model Mech 8(7): 657–667
23. Alexandrov PN, Zhao Y, Pogue AI, Tarr MA, Kruck TP, Percy ME, Cui JG, Lukiw WJ (2005) Synergistic effects of iron and aluminum on stress-related gene expression in primary human neural cells. J Alzheimers Dis 8(2):117–127
24. Pogue AI, Percy ME, Cui JG, Li YY, Bhattacharjee S, Hill JM, Kruck TP, Zhao Y et al (2011) Up-regulation of NF-κB-sensitive miRNA-125b and miRNA-146a in metal sulfate-stressed human astroglial (HAG) primary cell cultures. J Inorg Biochem 105(11): 1434–1437
25. Everett J, Céspedes E, Shelford LR, Exley C, Collingwood JF, Dobson J, van der Laan G, Jenkins CA et al (2014) Ferrous iron formation following the co-aggregation of ferric iron and the Alzheimer's disease peptide β-amyloid (1-42). J R Soc Interface 11(95):20140165
26. House MJ, St Pierre TG, Kowdley KV, Montine T, Connor J, Beard J, Berger J, Siddaiah N et al (2007) Correlation of proton transverse relaxation rates (R2) with iron concentrations in postmortem brain tissue from Alzheimer's disease patients. Magn Reson Med 57(1): 172–180
27. Connor JR, Menzies SL, St Martin SM, Mufson EJ (1992) A histochemical study of iron, transferrin, and ferritin in Alzheimer's diseased brains. J Neurosci Res 31(1):75–83
28. Bush AI (2013) The metal theory of Alzheimer's disease. J Alzheimers Dis 33(Suppl 1):S277–S281
29. van Bergen JM, Li X, Hua J, Schreiner SJ, Steininger SC, Quevenco FC, Wyss M, Gietl AF et al (2016) Colocalization of cerebral iron with amyloid β in mild cognitive impairment. Sci Rep 6:35514
30. Nagai K, Jiang MH, Hada J, Nagata T, Yajima Y, Yamamoto S, Nishizaki T (2002) (−)-Epigallocatechin gallate protects against NO stress-induced neuronal damage after ischemia by acting as an antioxidant. Brain Res 956(2):319–322
31. Sai K, Yang D, Yamamoto H, Fujikawa H, Yamamoto S, Nagata T, Saito M, Yamamura T et al (2006) A1 adenosine receptor signal and AMPK involving caspase-9/−3 activation are responsible for adenosine-induced RCR-1 astrocytoma cell death. Neurotoxicology 27(4):458–467
32. Jin Y, Tsuchiya A, Kanno T, Nishizaki T (2015) Amyloid-β peptide increases cell surface localization of α7 ACh receptor to protect neurons from amyloid β-induced damage. Biochem Biophys Res Commun 468(1–2):157–160
33. Nussbaum JM, Seward ME, Bloom GS (2013) Alzheimer disease: a tale of two prions. Prion 7(1):14–19
34. Chafekar SM, Zwart R, Veerhuis R, Vanderstichele H, Baas F, Scheper W (2008) Increased $A\beta_{1\text{-}42}$ production sensitizes neuroblastoma cells for ER stress toxicity. Curr Alzheimer Res 5(5):469–474
35. Abe K, Saito H (1998) Amyloid β protein inhibits cellular MTT reduction not by suppression of mitochondrial succinate dehydrogenase but by acceleration of MTT formazan exocytosis in cultured rat cortical astrocytes. Neurosci Res 31(4):295–305
36. Fu W, Ruangkittisakul A, MacTavish D, Shi JY, Ballanyi K, Jhamandas JH (2012) Amyloid β (Aβ) peptide directly activates amylin-3 receptor subtype by triggering multiple intracellular signaling pathways. J Biol Chem 287(22):18820–18830
37. Jhamandas JH, Li Z, Westaway D, Yang J, Jassar S, MacTavish D (2011) Actions of β-amyloid protein on human neurons are expressed through the amylin receptor. Am J Pathol 178(1): 140–149
38. Jhamandas JH, Mactavish D (2012) β-Amyloid protein (Aβ) and human amylin regulation of apoptotic genes occurs through the amylin receptor. Apoptosis 17(1):37–47
39. Fu W, Vukojevic V, Patel A, Soudy R, MacTavish D, Westaway D, Kaur K, Goncharuk V et al (2017) Role of microglial amylin receptors in mediating beta amyloid (Aβ)-induced inflammation. J Neuroinflammation 14(1):199
40. Qiu WQ (2017) Amylin and its G-protein-coupled receptor: a probable pathological process and drug target for Alzheimer's disease. Neuroscience 356:44–51
41. Fu W, Patel A, Kimura R, Soudy R, Jhamandas JH (2017) Amylin receptor: a potential therapeutic target for Alzheimer's disease. Trends Mol Med 23(8):709–720