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Amyloid β-peptide preconditioning reduces glutamate-induced neurotoxicity by promoting endocytosis of NMDA receptor

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Abstract

Amyloid β-peptide (Aβ) and glutamate are generally believed to be closely related to the pathogenesis of Alzheimer's disease and cerebrovascular disease, respectively. Recent advances suggest that risk factors linked to cerebrovascular disease significantly increase the risk of developing Alzheimer's disease. In this study, we examined the effects of pretreatment of cultured hippocampal neurons with Aβ(1–42) (0.3, 0.5, and 1.0 μM) for 3 h (Aβ preconditioning) on glutamate-induced neurotoxicity. Aβ preconditioning significantly reduced both glutamate-induced neurotoxicity and the glutamate-induced increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Aβ preconditioning significantly reduced cell surface expression of *N*-methyl-D-aspartate (NMDA) glutamate receptor subunit protein NR1, although it exerted no significant effect on the total expression of NR1. These results suggest that Aβ preconditioning reduced glutamate-induced neurotoxicity by promoting endocytosis of NMDA receptor, followed by inhibition of the increase in $[Ca^{2+}]_i$. Our results support the notion of an association between Alzheimer's disease and cerebrovascular disease, and suggest a new mechanism for neuroprotection by promoting endocytosis of NMDA receptor.

Keywords: Alzheimer's disease; Amyloid β-peptide; Cell death; Glutamate; Hippocampus; NMDA receptor; Preconditioning

Alzheimer's disease is a neurodegenerative disorder characterized clinically by cognitive impairment and pathologically by the appearance of senile plaques and neurofibrillary tangles [1]. The major component of the senile plaques is amyloid β-peptide (Aβ), which is a 39–43 amino acid peptide fragment derived from amyloid precursor protein. Although the exact etiology of Alzheimer's disease remains to be determined, it has been proposed that accumulation of Aβ is closely related to the pathogenesis of Alzheimer's disease. In addition to Aβ, enhancement of excitotoxicity by glutamate has also been implicated in the pathogenesis of Alzheimer's disease [2]. Several studies have demonstrated that Aβ exacerbates glutamate-induced neuronal cell death [3,4], and inhibition of glutamate receptor reduces Aβ-induced neuronal cell death [5,6]. These results suggest that the interaction between Aβ- and glutamate-induced neuronal cell death is critical for the pathogenesis of Alzheimer's disease.

Ischemic preconditioning is an endogenous neuroprotective mechanism by which sublethal ischemic events render a tissue more tolerant to subsequent lethal ischemic events. In ischemic events, overactivation of NMDA glutamate receptor is widely believed to be the main signaling pathway to cell injury. Subtoxic preconditioning levels of NMDA protect neurons against excitotoxicity induced by toxic levels of glutamate [7–9]. A recent study demonstrated that hypoxia causes an increase of the expression level of amyloid precursor protein, a decrease in α-secretase activity, and a decrease of the expression levels of two

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Aβ degrading enzymes, neprilysin and endothelin-converting enzyme, altogether leading to the accumulation of neurotoxic Aβ. Interestingly, hypoxia preconditioning restored the expression levels of amyloid precursor protein and two Aβ degrading enzymes, and the activity level of α-secretase, to their control values [10]. These results suggest that ischemic preconditioning reduces Aβ-induced neurotoxicity.

Based on the evidence mentioned above, we hypothesized that Aβ preconditioning protects neurons against glutamate-induced cell death. We first ascertained the experimental conditions for Aβ preconditioning in hippocampal cultures. Next we examined the effects of Aβ preconditioning on glutamate-induced neuronal cell death. Finally, we examined a possible mechanism underlying the neuroprotective effect of Aβ preconditioning on glutamate-induced neuronal cell death.

Materials and methods

Reagents. Neurobasal medium, B27 supplement, glutamine, horse serum, penicillin, and streptomycin were purchased from Invitrogen Corp. (Carlsbad, CA, USA). Aβ(1–42) was from Peptide Institute Inc. (Osaka, Japan). Fura 2-AM was from Dojindo Laboratories (Kumamoto, Japan). The Cytotoxicity Detection LDH kit was from Kyokuto Pharmaceutical Industrial Corp. (Tokyo, Japan). All other reagents were purchased from Sigma (St. Louis, MO, USA) or Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Preparation of Aβ(1–42). Aβ(1–42) was dissolved in 0.1% ammonia in water just before use according to the manufacturer's instruction. There was no attempt to induce fibril formation or aging of peptide solutions prior to their use.

Neuronal cell cultures. The use of experimental animals in this study was conducted in accordance with the ethical guidelines of the Kyoto University Animal Experimentation Committee and the guidelines of the Japanese Pharmacological Society. Hippocampal neurons were prepared from 15-day-old embryonic ICR mice (Nihon SLC, Shizuoka, Japan). Briefly, hippocampi were dissected and dissociated with 0.2% trypsin in phosphate-buffered saline (PBS). Dissociated cell suspensions were plated at 5.0×10^5 cells/cm^2 on plastic tissue culture dishes coated with polyethylenimine in defined medium (Neurobasal/B27) supplemented with 2 mM glutamine, penicillin, and streptomycin. Cells were cultured for 6 days *in vitro* before experiments at 37 °C in a 5% CO_2/95% air humidified incubator.

Lactate dehydrogenase (LDH) release assay. Cytotoxicity was assessed by detecting the leakage of LDH into the medium. The amount of LDH was determined using a Cytotoxicity Detection LDH kit according to the manufacturer's instructions. In this assay, NAD is reduced to NADH through the conversion of lactate to pyruvate by LDH, and NADH reduces tetrazolium into formazan dyes in the presence of diaphorase. Briefly, 50 μl of culture supernatant was mixed with 50 μl of the LDH substrate mixture in a 96-well plate. After incubation for 45 min at room temperature, the reaction was stopped by adding 100 μl of 1 N HCl, and the absorbance at 570 nm was determined using a microplate reader (Model 680, Bio-Rad, Hercules, CA, USA). The background absorbance obtained from the culture medium was subtracted. Cytotoxicity was expressed as a percentage of the amount of LDH from the culture medium of hippocampal neurons treated with no treatment (Sham).

Intracellular Ca^{2+} imaging. The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured with a Ca^{2+}-sensitive fluorescent dye, fura 2-acetoxymethylester (fura 2-AM), using a fluorescence imaging system (ARGUS/HiSCA, Hamamatsu Photonics K.K., Shizuoka, Japan), according to the method described previously [11]. Cultured hippocampal neurons on glass coverslips were incubated in Krebs–Ringer buffer (137 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 25 mM D-(+)-glucose, and 10 mM Hepes, pH 7.4) containing 5 μM fura 2-AM and 0.01% cremophor EL for 1 h at 37 °C and then rinsed with the buffer. A coverslip was mounted on a recording chamber placed under a fluorescent microscope. Cells were alternatively illuminated with lights (wavelengths of 340 and 380 nm) at an interval of 2 s, and the emission was measured at 500 nm. Fluorescence imaging was performed for 10 min at room temperature. The peak amplitude of the fluorescence ratio (340/380 nm) just after glutamate application was adapted as an index of glutamate-induced Ca^{2+} influx.

Surface biotinylation assay. Cultured hippocampal neurons were placed on ice, rinsed in cold PBS, and incubated in PBS containing 1.0 mg/ml Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL, USA) for 30 min at 4 °C. After incubation, neurons were rinsed three times in PBS and lysed in 150 μl PBS containing complete proteases (Nacalai Tesque, Kyoto, Japan), phosphatase inhibitor cocktail (Sigma), 0.1% sodium dodecyl sulfate (SDS), and 1% Triton X-100. To detect the total expression of NR1, 10% of the cell lysate was boiled at 95 °C for 5 min in SDS-containing-buffer with 5% β-mercaptoethanol. To detect the surface expression of NR1, 80% of the cell lysate was incubated with streptavidin gel (Pierce, Rockford, IL, USA).

Western blotting. Aliquots of 10 μg of protein were loaded on a 4/20 gradient SDS–polyacrylamide gel (PAG Mini DAIICHI, Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan) for electrophoresis at a constant current of 40 mA/plate for 1 h at room temperature and subsequent blotting to a nitrocellulose polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA, USA) pretreated with 100% methanol. After blocking by 5% skim milk, the membrane was incubated with antibody against NR1 (1:1000, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or GAPDH (1:10,000, Ambion, Austin, TX, USA) diluted with the buffer containing 5% skim milk, followed by a reaction with an anti-goat IgG or anti-mouse IgG antibody conjugated with peroxidase. Proteins reactive with the antibodies were visualized using the ECL system (Amersham Biosciences, Buckinghamshire, UK) on X-Omat Blue films (Kodak, Tokyo, Japan). Densitometric determination was carried out using the NIH image and normalized to each GAPDH internal control.

Statistical analysis. Statistical comparisons were made by Student's *t*-test or one-way analysis of variance followed by Dunnett's multiple comparison test using SPSS version 12.0 (SPSS Inc., Chicago, IL). Results were considered significant at $P < 0.05$.

Results

Short-term Aβ(1–42) treatment

Cultured hippocampal neurons were incubated with Aβ(1–42) for 48 h. Cell death was assessed by the LDH release assay. Exposure to Aβ(1–42) for 48 h caused an increase in cell death in a concentration-dependent manner (Fig. 1A). A significant increase in cell death was observed at 1 μM or higher concentration of Aβ(1–42). Neurite degeneration and a reduction in the number of cell bodies were also observed (Fig. 1B). These results are consistent with previous findings that administration of Aβ(1–42) is neurotoxic *in vitro* and *in vivo* [12–15]. In contrast, exposure to Aβ(1–42) for 3 h caused no significant effect on cell viability or cell morphology at concentrations ranging from 0.1 to 3 μM (Fig. 1C and D).

Effects of Aβ(1–42) preconditioning on glutamate-induced neurotoxicity

The overactivation of NMDA glutamate receptor is widely believed to be involved in cell death in response

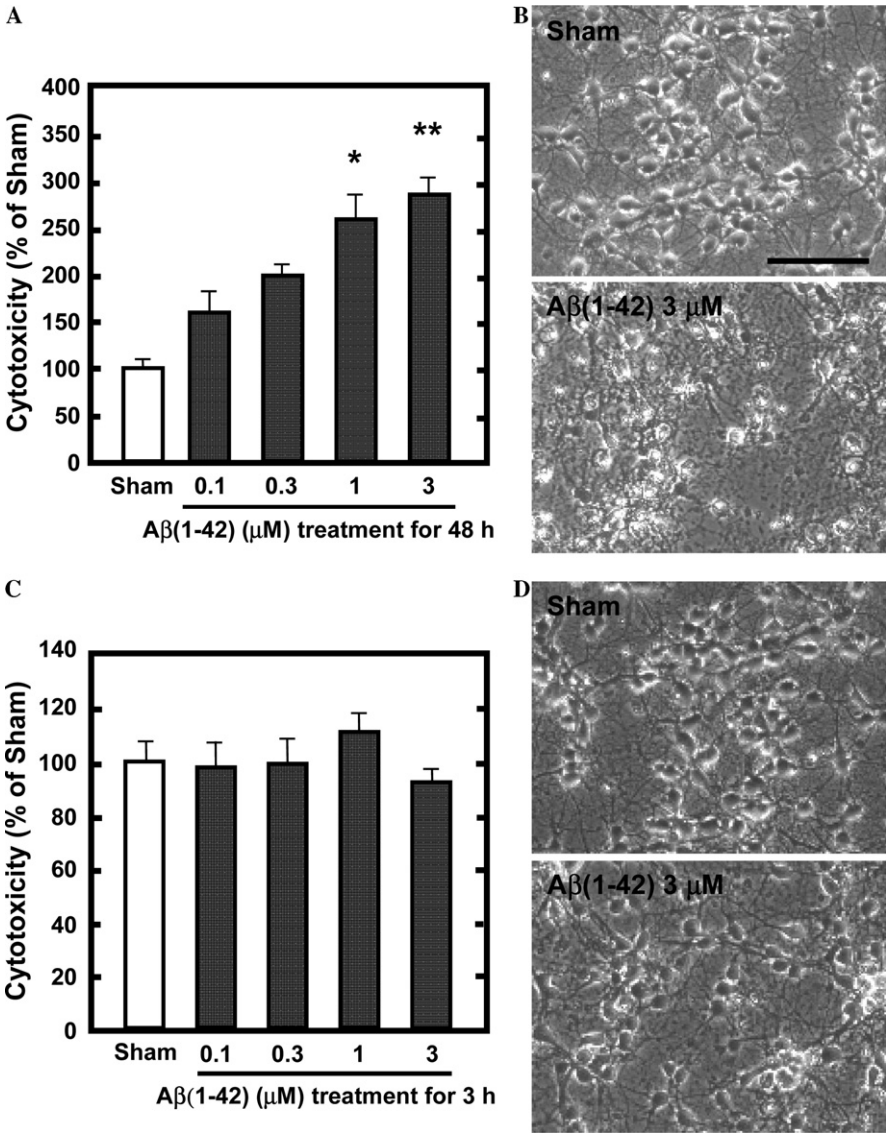

Fig. 1. Aβ(1–42)-induced cell toxicity in mouse cultured hippocampal neurons. Cytotoxicity induced by Aβ(1–42) for 48 h (A) or 3 h (C). The LDH release assay was conducted after a 3-h or 48-h exposure to Aβ(1–42). The open column represents cells to which Aβ(1–42) was not added (Sham). Each value represents the mean ± SEM ($n = 5$–6). $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. Sham. Representative phase contrast photomicrographs showing hippocampal neurons exposed to vehicle or Aβ(1–42) (3 μM) for 48 h (B) or 3 h (D). Scale bar for all: 100 μm.

to ischemic injury. In addition, NMDA preconditioning has also been reported to reduce glutamate-induced neurotoxicity [7]. Therefore, we investigated the possibility that Aβ(1–42) preconditioning protects hippocampal cells against glutamate- or Aβ(1–42)-induced neurotoxicity. Cultured hippocampal neurons were preincubated with Aβ(1–42) (0.3, 0.5, or 1.0 μM) for 3 h, and cell death was induced by the addition of glutamate (3, 10, or 30 μM) for 24 h. When glutamate at 3 or 10 μM was added to cultured hippocampal neurons for 24 h, Aβ(1–42) preconditioning (0.3, 0.5 or, 1.0 μM) significantly reduced the cell death (Fig. 2A and B). The cytotoxicity induced by glutamate at 30 μM was severer than that induced by glutamate at 3 or 10 μM. Aβ(1–42) preconditioning (0.5 or 1.0 μM) significantly reduced the cell death induced by glutamate at 30 μM (Fig. 2C). Next the effect of Aβ(1–42) preconditioning on the cell death induced by Aβ(1–42) was examined. Cultured hippocampal neurons were preincubated with Aβ(1–42) (0.3, 0.5, or 1.0 μM) for 3 h, and cell death was induced by the addition of Aβ(1–42) (1 μM) for 48 h. Exposure to Aβ(1–42) (1 μM) for 48 h caused a significant increase in cell death. Unlike glutamate-induced neurotoxicity, Aβ(1–42) preconditioning at 0.3 or 0.5 μM caused no significant effect on Aβ(1–42)-induced neurotoxicity (Fig. 2D). Conversely, Aβ(1–42) preconditioning at 1 μM exacerbated Aβ(1–42)-induced neurotoxicity. This is not surprising because cell death was induced by the addition of Aβ(1–42) (1 μM) for 51 h under these experimental conditions.

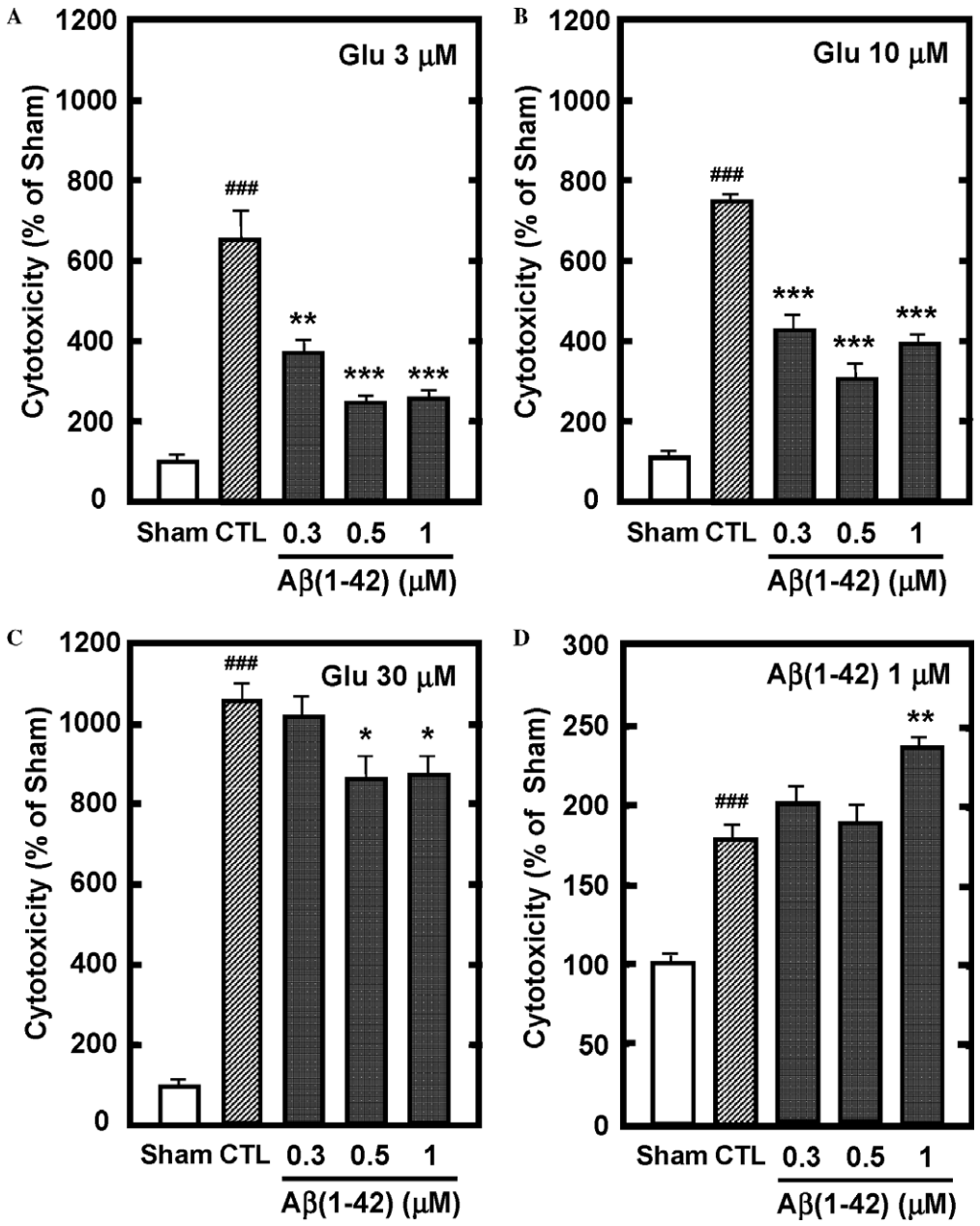

Fig. 2. Effects of Aβ(1–42) preconditioning on glutamate- or Aβ(1–42)-induced neurotoxicity. Cultured hippocampal neurons were preincubated with Aβ(1–42) (0.3, 0.5, or 1.0 μM) for 3 h, and cell death was induced by glutamate at 3 μM (A), 10 μM (B), or 30 μM (C) for 24 h. The LDH release assay was conducted after a 24-h exposure to glutamate. Cultured hippocampal neurons were preincubated with Aβ(1–42) (0.3, 0.5, or 1.0 μM) for 3 h, and cell death was induced by 1 μM Aβ(1–42) for 48 h (D). The LDH release assay was conducted after a 48-h exposure to 1 μM Aβ(1–42). The open column represents cells with no treatment (Sham). The hatched column represents cells with no Aβ(1–42) pretreatment (CTL). Each value represents the mean ± SEM ($n = 5$–6). $^{###}P < 0.001$ vs. Sham. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. CTL.

Mechanism of neuroprotective effect of Aβ(1–42) preconditioning

As mentioned above, Aβ(1–42) preconditioning showed a neuroprotective effect against glutamate-induced neurotoxicity. However, the mechanism causing the neuroprotective effect of Aβ(1–42) preconditioning remains unknown. Since it is generally believed that the overactivation of NMDA glutamate receptor causes excessive increase in $[Ca^{2+}]_i$ and this correlates well with subsequent cell death [16], we evaluated the effect of Aβ(1–42) preconditioning on the glutamate-induced increase in $[Ca^{2+}]_i$ using fura 2 fluorescence imaging. Glutamate at 10 μM induced a remarkable increase in $[Ca^{2+}]_i$ (Fig. 3A). This increase was mainly due to Ca^{2+} influx through NMDA receptor, as indicated by the fact that an NMDA receptor antagonist, MK-801, substantially reduced the glutamate-induced increase in $[Ca^{2+}]_i$ (data not shown). Cultured hippocampal neurons were preincubated with Aβ(1–42) (1 μM) for 3 h, and an increase in $[Ca^{2+}]_i$ was induced by the addition of

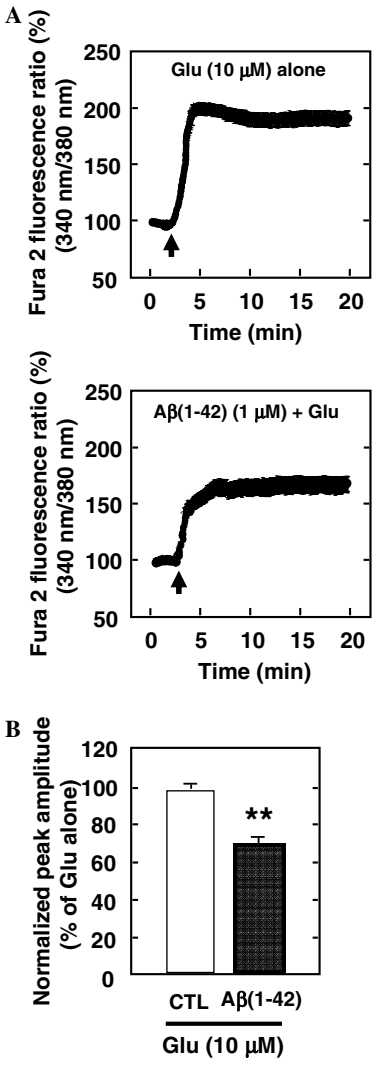

Fig. 3. Effect of Aβ(1–42) preconditioning on glutamate-induced increases in $[Ca^{2+}]_i$. (A) Representative fluorescence ratio obtained with glutamate alone (upper) and glutamate plus Aβ(1–42) pretreatment for 3 h (lower). Arrows indicate the application of glutamate. (B) Normalized peak amplitude for glutamate alone (CTL) and glutamate plus Aβ(1–42) pretreatment for 3 h. Each value represents mean ± SEM ($n = 30$–40). $^{**}P < 0.01$ vs. glutamate alone.

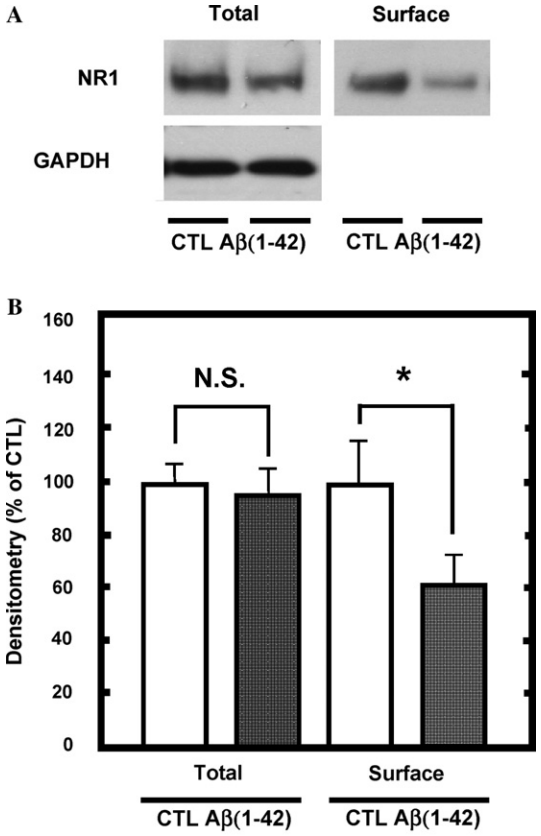

Fig. 4. Aβ(1–42)-induced endocytosis of NMDA receptor subunit protein NR1. (A) Cultured hippocampal neurons were incubated in the absence (CTL) or presence of Aβ(1–42) (1 μM) for 3 h, and cell surface expression of NR1 was measured by biotinylation. GAPDH was used as an internal control. (B) Quantification of biotinylation immunoblotting. Each value represents mean ± SEM ($n = 3$–4). N.S.; not significant, $^{*}P < 0.05$.

glutamate (10 μM) (Fig. 3A). Aβ(1–42) preconditioning significantly reduced the glutamate-induced increase in $[Ca^{2+}]_i$ to 72.6 ± 2.9% compared to the control level (Fig. 3B). These results suggest that Aβ(1–42) preconditioning exerts neuroprotective effects by reducing Ca^{2+} influx through NMDA receptor. A recent study demonstrated that Aβ reduces glutamatergic transmission by promoting endocytosis of NMDA receptor in cultured cortical neurons [17]. Therefore, we examined the effect of Aβ(1–42) preconditioning on endocytosis of NMDA receptor. Cultured hippocampal neurons were preincubated with Aβ(1–42) (1 μM) for 3 h, and cell surface expression of NMDA receptor subunit protein NR1 was quantified using biotinylation. Aβ(1–42) preconditioning significantly reduced the surface expression of NR1 to 60.9 ± 16.8% compared to the control level (Fig. 4). On the other hand, Aβ(1–42) preconditioning exerted no significant effect on the total expression of NR1. These results suggest that inhibition of the glutamate-induced increase in $[Ca^{2+}]_i$ by Aβ(1–42) preconditioning is due to the promotion of NMDA receptor endocytosis.

Discussion

Epidemiological studies have demonstrated that risk factors linked to cerebrovascular disease such as stroke significantly increase the risk of developing Alzheimer's disease [18,19]. More than one-third of patients with Alzheimer's disease exhibit variable cerebrovascular pathology, including cerebral amyloid angiopathy, microvascular degeneration, and cerebral infarct. Stroke events are known to worsen the cognitive function of patients diagnosed with Alzheimer's disease [20]. Thus, several lines of evidence suggest that there is a strong association between Alzheimer's disease and cerebrovascular disease. However, the exact nature of the relationship between these two diseases is not well understood.

It is generally believed that Aβ and glutamate are closely related to the pathogenesis of Alzheimer's disease and cerebrovascular disease such as stroke, respectively. In this study, we explored the possibility that Aβ preconditioning can regulate the glutamate-induced neuronal cell death. Treatment of hippocampal neurons with Aβ(1–42) for 48 h increased cytotoxicity in a concentration-dependent manner, whereas no cytotoxicity was observed after treatment with Aβ(1–42) for 3 h (Fig. 1). Thus we concluded the appropriate experimental condition for Aβ preconditioning was treatment with Aβ(1–42) for 3 h, which is in accord with the fact that a brief ischemic episode induces protection against a subsequent severe ischemic insult. We found that Aβ(1–42) preconditioning protected hippocampal neurons against glutamate-induced cell death (Fig. 2A–C). A recent study demonstrated that hypoxia modifies the metabolism of amyloid precursor protein, leading to the accumulation of neurotoxic Aβ. Interestingly, hypoxic preconditioning appears to exert a neuroprotective effect by restoring the metabolism of amyloid precursor protein [10]. Together with our results, these observations support the notion that there is strong association between Alzheimer's disease and cerebrovascular disease. On the other hand, Aβ(1–42) preconditioning failed to protect hippocampal neurons against Aβ(1–42)-induced cell death (Fig. 2D). Although the precise mechanism of Aβ-induced cell death is not well understood, it is speculated that Aβ neurotoxicity is due to various factors, including oxidative stress, excessive increase in $[Ca^{2+}]_i$, and glutamate accumulation [21–23]. Aβ(1–42) preconditioning alone may not be sufficient to inhibit these deleterious events. Furthermore, we found that Aβ(1–42) preconditioning reduced glutamate-induced increased in $[Ca^{2+}]_i$ by promoting endocytosis of NMDA receptor (Figs. 3 and 4). Postsynaptic α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) glutamate receptors have been reported to be internalized via a clathrin-dependent mechanism [24], whereas NMDA glutamate receptors are expressed stably at the postsynaptic membrane under normal conditions [25]. In addition, postsynaptic density-95 (PSD-95), a multivalent synaptic scaffolding protein and core component of the postsynaptic density at excitatory synapses, has been reported to enhance the stability of NMDA receptor at the postsynaptic membrane by inhibiting NMDA receptor subunit protein NR2-dependent internalization [26,27]. Since Aβ induces the degradation of PSD-95 via activation of NMDA receptor [28], it is possible that Aβ preconditioning promotes the endocytosis of NMDA receptor, which is composed of NR1 and NR2, by the degradation of PDS-95.

Ischemic preconditioning has been reported to be an endogenous neuroprotective mechanism against subsequent lethal ischemia. Like ischemic preconditioning, Aβ preconditioning may be an endogenous neuroprotective mechanism against neurodegeneration in the pathogenesis of Alzheimer's disease, because Aβ preconditioning can reduce glutamate-induced neuronal cell death. In cultured cortical astrocytes, Aβ enhances the clearance of extracellular glutamate by promoting the expression of glutamate transporter GLAST [29]. Thus it is possible that Aβ preconditioning inhibits glutamate-induced exacerbation of Alzheimer's disease by protecting neurons against glutamate-induced cell death in neurons and by promoting the clearance of extracellular glutamate in astrocytes.

In conclusion, this is the first report showing that Aβ preconditioning protects hippocamapal neurons against glutamate-induced cell death. Our results provide fundamental insights that should be useful for therapeutic interventions for Alzheimer's disease.

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