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Toxicity in Rat Primary Neurons through the Cellular Oxidative Stress Induced by the Turn Formation at Positions 22 and 23 of Aβ42

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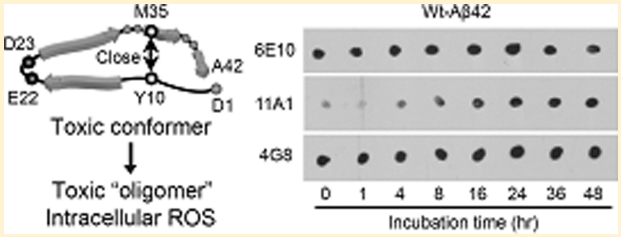
Supporting Information

ABSTRACT: The 42-mer amyloid β-protein (Aβ42) aggregates to form soluble oligomers that cause memory loss and synaptotoxicity in Alzheimer's disease (AD). Oxidative stress is closely related to the pathogenesis of AD. We previously identified the toxic conformer of Aβ42 with a turn at positions 22 and 23 ("toxic turn") by solid-state NMR and demonstrated that a monoclonal antibody (11A1) against the toxic turn in Aβ42 mainly detected the oligomer in the brains of AD patients. Our recent study suggested that oxidative stress is a key factor of the oligomerization and cognitive impairment induced by Aβ overproduction in vivo. However, the involvement of the toxic conformer in Aβ42-induced oxidative damage remains unclear. To investigate this mechanism, we examined the levels of intracellular reactive oxygen species (ROS) and neurotoxicity in rat primary neurons using E22P-Aβ42, a mutant that induces a turn at positions 22 and 23, and E22V-Aβ42, a turn-preventing mutant. E22P-Aβ42, but not E22V-Aβ42, induced greater ROS production than Wt-Aβ42 in addition to potent neurotoxicity. Interestingly, the formation of the toxic conformer in both E22P-Aβ42 and Wt-Aβ42 probed by the 11A1 antibody preceded Aβ42-induced neurotoxicity. Trolox (a radical scavenger) and Congo red (an aggregation inhibitor) significantly prevented the neurotoxicity and intracellular ROS induced by E22P-Aβ42 and Wt-Aβ42, respectively. These results suggest that Aβ42-mediated toxicity is caused by the turn that favors toxic oligomers, which increase generation of ROS.



KEYWORDS: *Alzheimer's disease, amyloid β, toxic conformer, turn structure, oxidative stress, neurotoxicity*

Alzheimer's disease (AD) is a progressive neurodegenerative disease characterized by the deposition of amyloid fibrils.[1] The deposits are mainly composed of 40- and 42-mer amyloid β-proteins (Aβ40 and Aβ42, respectively), which are produced from the amyloid β-protein precursor by two proteases, β- and γ-secretase.[2] Aβ42 is considered to play a pivotal role in the pathogenesis of AD because it more strongly aggregates through β-sheet transformation and is more neurotoxic than Aβ40.[3] A number of studies have indicated that oligomeric assemblies of Aβ are involved in synaptic dysfunction.[4,5] Conversely, oxidative stress is a key contributing factor to AD progression.[6] Aβ-induced neurotoxicity is linked to its radicalization in vitro.[7,8]

Our previous investigation using solid-state NMR together with systematic proline replacement distinguished the toxic conformer, with a turn at positions 22 and 23, in Aβ42 aggregates from the nontoxic conformer, with a turn at positions 25 and 26 (Figure 1).[9] To date, we have been developing a model for oligomerization of the toxic conformer. A "toxic" turn would bring the Y10 close to M35 to produce the S-oxidized radical cation, which could be stabilized by a C-terminal hydrophobic core formed by the intramolecular β-sheet at positions 38 and 39, resulting in oligomer formation.[8] The toxic conformer is potently aggregative and exhibits cytotoxicity in PC12 cells.[9−11] Furthermore, a monoclonal antibody (11A1) targeting the toxic turn in Aβ42 mainly recognized Aβ oligomers (trimers) in the brains of human AD patients.[12] Recently, Murakami et al. demonstrated that cytoplasmic oxidative stress accelerated the oligomer formation of Aβ and memory loss in a transgenic mouse model of AD.[13] However, whether the toxic conformer of Aβ42 contributes to cellular oxidative damage remains to be elucidated, although several Aβ42 mutants with toxic turns possessed potent ability to produce radicals in cell-free experiments.[8]

This paper describes the contribution of the toxic conformer of Aβ42 to its oligomerization and induction of intracellular oxidative stress in rat primary neurons. We used Aβ42 mutants (E22P, E22K, E22V, and G25P), as proline and lysine are often found in the turn structure, whereas they rarely exist in β-sheets. In contrast, valine is known as a turn breaker.[14] E22P-Aβ42 potently induced intracellular reactive oxygen species (ROS) with subsequent cytotoxicity in neurons, and these effects were ameliorated by the antioxidant Trolox and the

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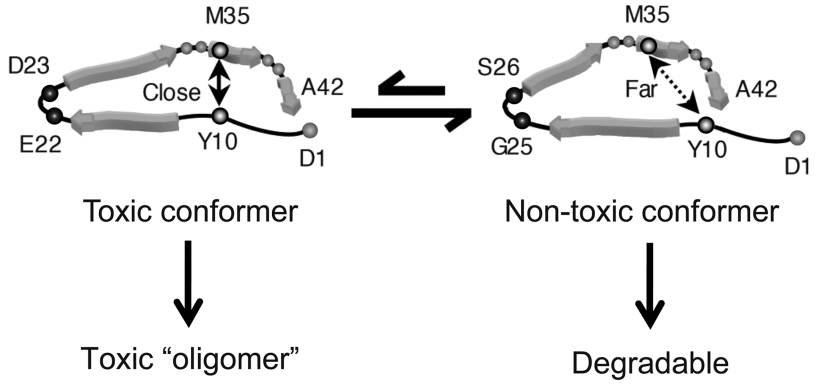

Figure 1. Two conformers of Aβ42 identified in solid-state NMR studies: (A) the toxic conformer with a turn at positions 22 and 23 and (B) the nontoxic conformer with a turn at positions 25 and 26. In the toxic conformer, a toxic turn would bring the Y10 close to M35 to radicalize M35, resulting in oligomer formation. On the other hand, the G25−S26 turn structure in the nontoxic one would not induce oligomer formation effectively. During the progression of AD, the equilibrium could shift to the toxic conformer under the condition of impaired metal homeostasis, and subsequently the toxic conformer could form the oligomer. Conversely, the nontoxic conformer can be degraded by Aβ proteases.

aggregation inhibitor Congo red. There was good agreement between the formation of toxic Aβ assemblies, as detected by the 11A1 antibody, and the neurotoxicity of E22P-Aβ42 and wild-type Aβ42 (Wt-Aβ42). These findings highlight a potential causative role for the formation of the toxic conformer of Aβ42 in Aβ42-induced intracellular ROS production.

RESULTS AND DISCUSSION

Neurotoxicity in a Primary Culture of Rat Cortical Neurons and Oligomer Formation of Aβ42 Mutants. To elucidate the cytotoxic effect of the toxic conformer of Aβ42 on rat primary neurons, cortical neuronal cultures were exposed to each Aβ42 mutant (20 μM) for 48 h, followed by the MTT assay. E22P- and E22K-Aβ42, which mimic the toxic conformer of Aβ42,[9] showed significant neurotoxicity compared with Wt-Aβ42 (Figure 2A). In contrast, E22V-Aβ42, in which the toxic turn is barely formed, was nontoxic. G25P-Aβ42, a mimic of the nontoxic conformer of Aβ42,[9] displayed slight neurotoxicity (Figure 2A).

E22P-Aβ42 enhanced the formation of the oligomer (trimer) in Western blotting under the condition of sodium dodecyl sulfate (SDS).[9] Because SDS can possibly affect the secondary structure of Aβ42,[15] dot blotting using the antitoxic conformer of Aβ42 antibody (11A1) which could detect Aβ oligomers in the brains of AD patients [12] was performed. As shown in Figure 2B, 11A1 strongly recognized E22P- and E22K-Aβ42, similar to Wt-Aβ42 recognition, whereas its immunoreactivity against E22V- and G25P-Aβ42 was weaker than that against Wt-Aβ42. Conversely, anti-Aβ1−17 antibody (6E10) similarly reacted to all proteins. The immunoreactivity of anti-Aβ17−24 (4G8) against the Aβ42 mutants at position 22 was weaker than that with Wt-Aβ42 and G25P-Aβ42. These results did not contradict the characterization of 4G8, in which the epitope specifically lies in the sequence of Aβ17−24 (i.e., the sequence in Wt-Aβ42 and G25P-Aβ42 is intact). Our previous studies using the double mutation of E22P,G25P-Aβ42 indicated that Aβ42 aggregates contain two different conformers: one with a turn at positions 22 and 23, the other with a turn at positions 25 and 26.[9] Because Aβ42 is in equilibrium between toxic and nontoxic conformers, E22V-Aβ42 and G25P-Aβ42 contain a slight amount of the toxic conformer. Considering the bright sensitivity of 11A1, it is not surprising that 11A1 against the toxic one slightly detected E22V-Aβ42 and G25P-Aβ42. These results are in good agreement with our previous enzyme immune assay of 11A1.[12] These findings suggest that the formation of the toxic conformer with the turn at positions 22 and 23 could be important for the neurotoxicity and oligomerization of Aβ42 and that E22P-Aβ42 is the most potent mimic of the toxic conformer of Aβ42.

It is reported that the Arctic mutant of Aβ42 (E22G-Aβ42) causes early onset AD. Thus, we examined the neurotoxicity and the toxic conformer formation of E22G-Aβ42 to investigate the correlation of the toxic conformer and early onset AD. E22G-Aβ42 induced as potent a neurotoxicity as E22P-Aβ42 and the toxic conformer formation (Supporting Information Figure 1). These findings suggest that toxic conformer formation might be important for the neurotoxicity induced by the mutant Aβ42, which is the cause of the early onset AD.

Time Course of the Neurotoxicity and Oligomerization of E22P-Aβ42. As a further investigation, the time dependency and concentration dependency of E22P-Aβ42 toxicity were tested. E22P-Aβ42 (20 μM) induced approximately 30% neurotoxicity after 16 h of treatment, and the extent of cell death exceeded 50% after treatment for 24 h (Figure 3A). E22P-Aβ42, even at 5 μM, induced significant cell death after 16 h of treatment. In contrast, Wt-Aβ42 was neurotoxic at 20 μM after 36 h of treatment but not after 24 h of treatment. After 8 h of incubation, the viability of all the treatments was almost 100% (Figure 3A). Notably, E22P-Aβ42 (1 μM) significantly induced toxicity in neurons after 48 h of treatment, whereas the toxicity of Wt-Aβ42 required at least 10 μM (Figure 3B). These results indicate that E22P-Aβ42 could induce neurotoxicity in a time- and concentration-dependent manner. The neurotoxicity induced by E22P-Aβ42 (5 μM) was similar to that of Wt-Aβ42 (20 μM); therefore, we compared the neurotoxicity of Wt-Aβ42 (20 μM) and E22P-Aβ42 (5 μM) in the following experiment.

In dot blotting of the Aβ42 mutants (20 μM) using the 11A1 antibody, the immunoreactivity of Wt-Aβ42 gradually increased and plateaued after 24 h of incubation, whereas the reactivity by other antibodies (6E10 and 4G8) did not drastically change over time (Figure 4A). As shown in Figure 4B, the velocity of the alteration of intensities in E22P-Aβ42 was substantially higher than that of Wt-Aβ42. It is worth noting that the toxic conformer of Aβ42 probed by 11A1 was not reactive with 4G8; these results were in good agreement with enzyme immunoassay data in a previous study.[12] It is not surprising to observe

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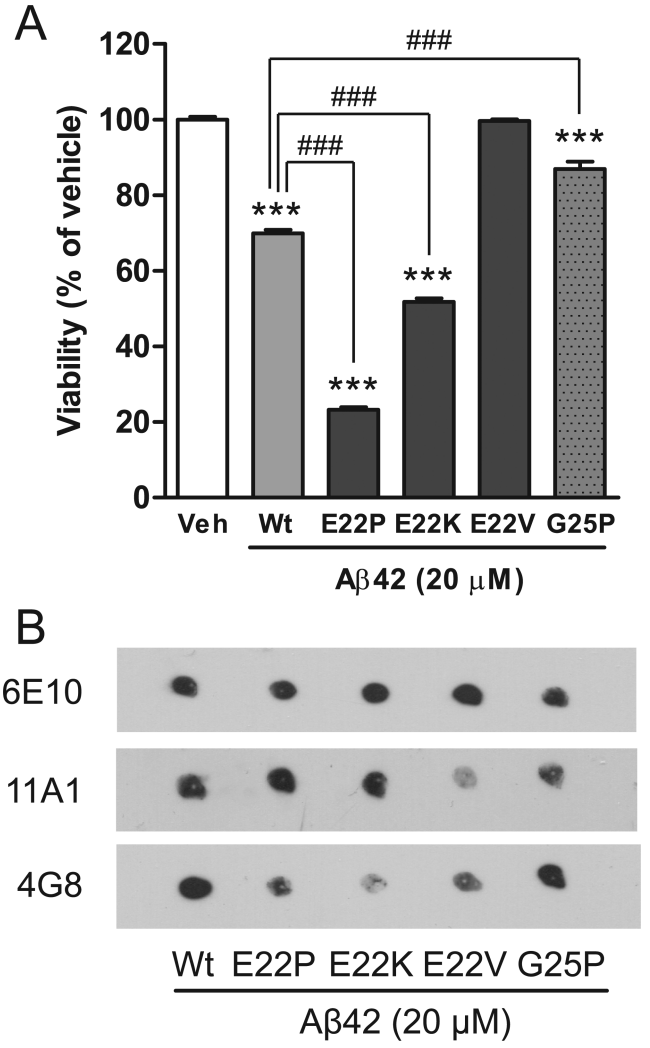

Figure 2. Neurotoxicity and oligomer formation of Aβ42 mutants. (A) The neurotoxic effects of Wt-, E22P-, E22K-, E22V-, and G25P-Aβ42 on neuronal cultures. Cultures were treated with Wt-Aβ42 or the mutants (20 μM) for 48 h. Cell viability was determined by the MTT assay: (***) $P < 0.001$ vs vehicle (Veh), ($^{###}$) $P < 0.001$. (B) Dot blotting of Aβ42 mutants detected by monoclonal antibodies: 6E10 (anti-Aβ1−17), 11A1 (antitoxic turn of Aβ22−23), and 4G8 (anti-Aβ17−24). Before spotting on the membrane, the solutions of Aβ42 mutants (20 μM) were incubated for 48 h at 37 °C.

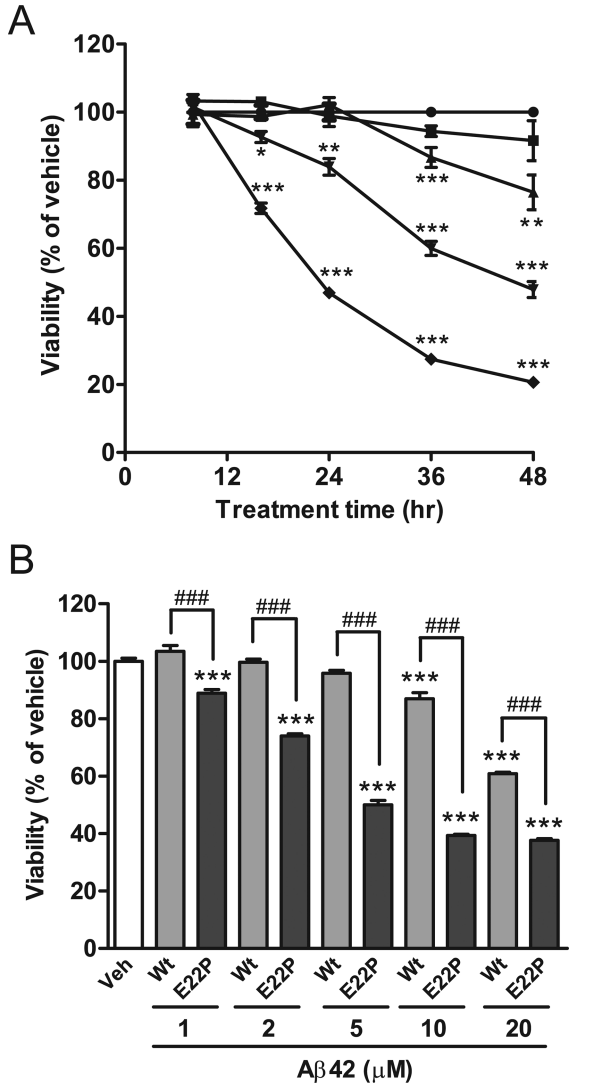

Figure 3. Time- and dose-dependency of the neurotoxicity and oligomerization of Wt- and E22P-Aβ42. (A) Time-dependency of the neurotoxicity induced by Wt- and E22P-Aβ42 (5 or 20 μM) treatment for 8, 16, 24, 36, and 48 h. The viability of all treatments was almost 100% for 8 h of incubation: ●, vehicle; ■, Wt-Aβ42 (5 μM); ▲, Wt-Aβ42 (20 μM); ▼, E22P-Aβ42 (5 μM); ◆, E22P-Aβ42 (20 μM); (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$ vs vehicle. (B) Dose-dependency of the neurotoxicity induced by Wt- and E22P-Aβ42 (1−20 μM) treatment for 48 h: (***) $P < 0.001$ vs vehicle, ($^{###}$) $P < 0.001$.

11A1-reactive oligomers even after 48 h because these results did not contradict previous studies by other groups; e.g., Aβ42 oligomers were still remaining after a 7-day incubation.[16,17] These findings imply that the oligomerization of the toxic conformer of Aβ42 could precede its neurotoxicity.

Production of Intracellular ROS Induced by E22P-Aβ42. Several reports suggest that intracellular oxidative stress is closely linked to the pathology of AD.[13] We examined the role of toxic conformation in the induction of intracellular ROS in neuronal cultures using the 2′,7′-dichlorofluorescein (DCF) assay in which cells were treated with 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) and its metabolite DCF is a fluorescent probe. A 4 h incubation with E22P-Aβ42 (5 and 20 μM) induced the increase of intracellular ROS at levels higher than Wt-Aβ42 (Figure 5B), although no difference was observed between E22P-Aβ42 and Wt-Aβ42 treatment after 1 h (Figure 5A). After 24 h of treatment, E22V-Aβ42 did not induce significant ROS generation (Figure 5C), indicating that the toxic turn in Aβ42 could contribute to the accumulation of cellular oxidative damage.

Protective Effects of Trolox and Congo Red against the Neurotoxicity and Production of Intracellular ROS Induced by E22P-Aβ42. The attenuation of neurotoxicity by inhibiting ROS generation or Aβ42 aggregation might be promising to suppress AD progression. There are many reports on the prevention of Aβ aggregation by antioxidative polyphenols or vitamins.[18] As shown in parts A and B of Figure 6, Trolox, a radical scavenger, decreased the cytotoxicity of Wt-Aβ42 and E22P-Aβ42. The extent of the inhibitory effect of Trolox on the neurotoxicity induced by E22P-Aβ42 was almost similar to that induced by Wt-Aβ42. The production of ROS in E22P-Aβ42- and Wt-Aβ42-treated cells was also abolished by Trolox treatment for 24 h (Figure 6C). These data

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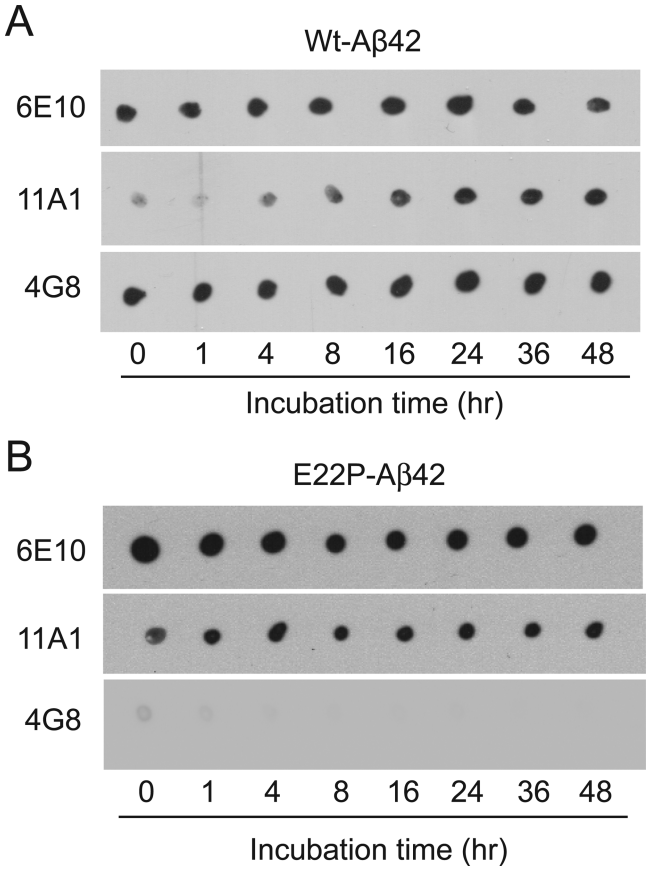

Figure 4. Dot blotting of Wt-Aβ42 and E22P-Aβ42. Detection was performed using the 6E10, 11A1, and 4G8 antibodies. The Wt- and E22P-Aβ42 (20 μM) solutions were incubated for each period at 37 °C.

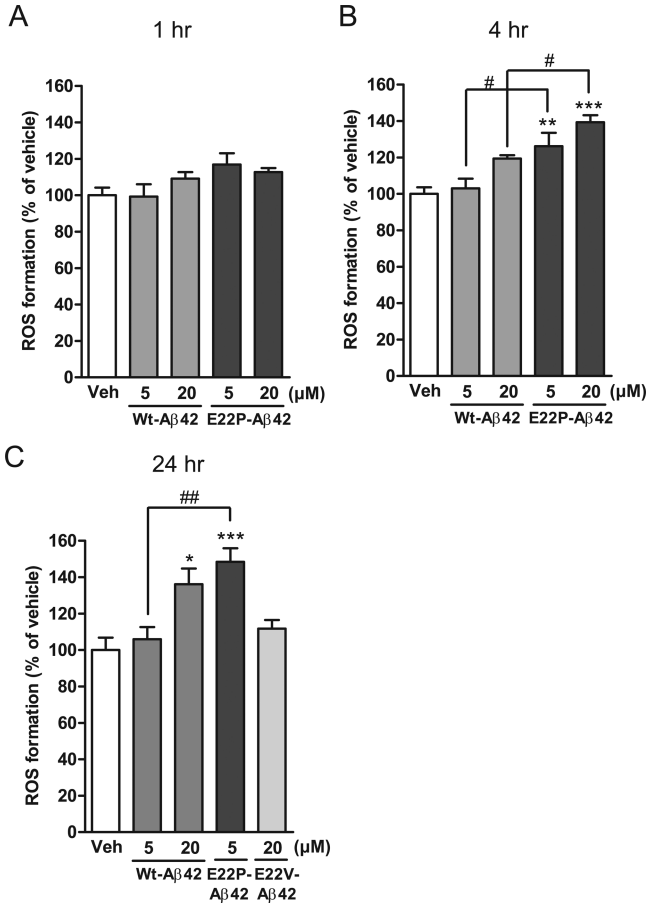

Figure 5. Induction of intracellular ROS by Wt- and E22P-Aβ42. Intracellular ROS levels were estimated by the DCF assay. Neuronal cultures were exposed to Wt- and E22P-Aβ42 (5 or 20 μM) for (A) 1, (B) 4, and (C) 24 h.: (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$ vs vehicle, ($^{\#}$) $P < 0.05$, ($^{\#\#}$) $P < 0.01$.

suggest that the intracellular ROS production induced by the toxic conformer of Aβ42 can elicit neurotoxicity.

Congo red is a conventional aggregation inhibitor because it has potent affinity for the β-sheet structure.[19] We confirmed that Congo red prevented the neurotoxicity of E22P-Aβ42 and Wt-Aβ42 (Figure 7A,B). Intriguingly, the intracellular oxidative stress induced by both Wt-Aβ42 and E22P-Aβ42 was significantly attenuated by Congo red (Figure 7C,D). Congo red (50 or 200 μM) alone had no effect on viability or ROS production. Together with these findings, it is suggested that the assembly of the toxic conformer of Aβ42 induces oxidative stress and neurotoxicity.

Intracellular Oxidative Stress in AD and E22P-Aβ42. The contribution of intracellular oxidative stress to AD pathogenesis has been suggested.[20,21] Nunomura and colleagues proposed the involvement of prominent RNA oxidation in the transition from normal aging to AD.[22,23] Murakami et al. reported that the deficiency of intracellular superoxide dismutase, one of the major antioxidative enzymes, promoted the generation of 8-hydroxydeoxyguanosine in DNA and Nε-carboxymethyl lysine in advanced glycation end products in vivo.[13] Recently, intracellular Aβ has received significant attention in the AD field. Ohyagi and colleagues revealed that intraneuronal Aβ accumulation and memory loss occur before extracellular Aβ deposition in mice.[24] Intracellular Aβ accumulation has been reported to frequently precede plaque formation in the human brain.[25] Considering reports that Aβ42 impairs the functions of organelles including mitochondria and ROS-generating enzymes such as nicotinamide adenine dinucleotide phosphate oxidase,[26−28] intraneuronal accumulation of Aβ42 could induce cellular oxidative stress.

In the current study, we demonstrate the potential contribution of the toxic turn structure in Aβ42 to the toxicity in rat primary neurons through cellular oxidative stress by using a mimic of the toxic conformer of Aβ42, E22P-Aβ42 (Figures 2, 3, and 5). In contrast, E22V-Aβ42, in which turn formation was blocked, did not induce any neurotoxicity or oxidative damage (Figures 2 and 5). This malfunction induced by the toxic conformer of Aβ42 was attenuated by the vitamin E-like radical scavenger Trolox and the conventional aggregation inhibitor Congo red (Figures 6 and 7). The preventive effect of Trolox on Aβ42-induced neurotoxicity was not as large as that on Aβ42-induced oxidative stress, implying that neurotoxicity is mediated by not only oxidative stress but also other mechanisms; e.g., Aβ42-induced abnormal calcium homeostasis and endoplasmic reticulum stress.[29−31] These findings lead to the conclusion that the formation of the toxic turn in Aβ42 plays a critical role in the intracellular ROS production.

Previous reports suggest that oxidative stress not only triggers neurotoxicity but also enhances Aβ oligomerization.[8,13] Because the trimer of E22P-Aβ42 was detected by Western blotting in the solution prepared without incubation,[9] the immunoreactivity of E22P- and E22K-Aβ42 (Figures 2 and 4) primarily revealed the formation of oligomers. In this study,

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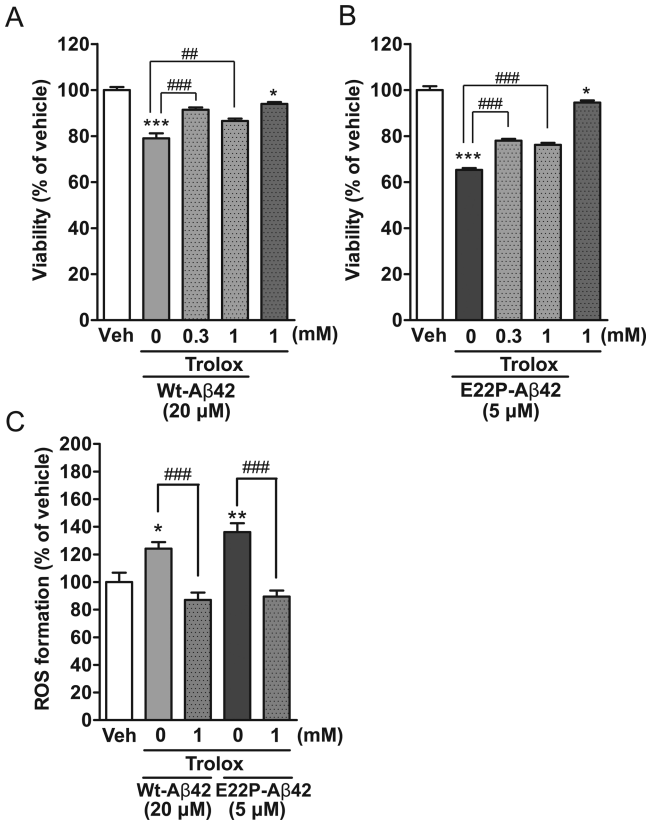

Figure 6. Protective effects of Trolox against Wt- and E22P-Aβ42-induced neurotoxicity and intracellular ROS accumulation. (A, B) In MTT test, cultures were treated with Trolox for 24 h before incubation only with (A) Wt-Aβ42 (20 μM) or (B) E22P-Aβ42 (5 μM) for 48 h. (C) In DCF assay, cultures were treated with Trolox for 24 h before treatment only with Wt-Aβ42 (20 μM) or E22P-Aβ42 (5 μM) for 24 h: (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$ vs vehicle, ($^{\#\#}$) $P < 0.01$, ($^{\#\#\#}$) $P < 0.001$.

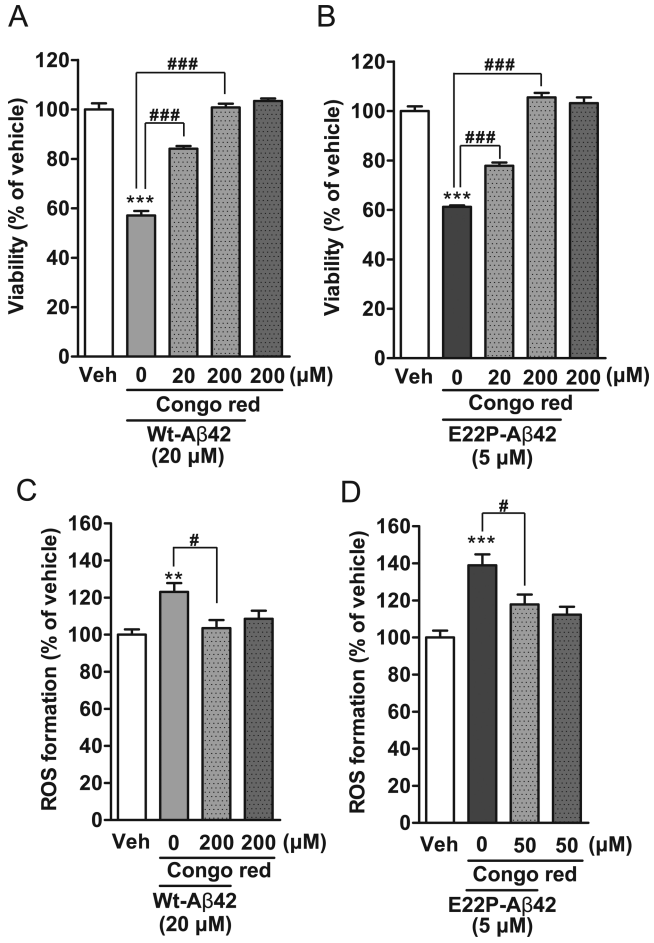

Figure 7. Preventive effects of Congo red against Wt-Aβ42 or E22P-Aβ42-induced neurotoxicity and intracellular ROS accumulation. (A, B) In MTT test, cultures were treated with Congo red and (A) Wt-Aβ42 (20 μM) or (B) E22P-Aβ42 (5 μM) for 48 h. (C, D) In DCF assay, cultures were incubated with Congo red and (C) Wt-Aβ42 (20 μM) or (D) E22P-Aβ42 (5 μM) for 24 h. The intrinsic fluorescence of Congo red was subtracted: (**) $P < 0.01$, (***) $P < 0.001$ vs vehicle, ($^{\#}$) $P < 0.05$, ($^{\#\#\#}$) $P < 0.001$.

E22P-Aβ42 enhanced the generation of intracellular ROS (Figure 5), and E22P-Aβ42 formed the toxic intermediate of aggregates more quickly than Wt-Aβ42 (Figure 4). These suggest that the excessive free radicals in the cytoplasm induced by the toxic turn in Aβ42 directly lead to Aβ42 oligomerization. Further investigation of the molecular mechanism would be required to clarify the involvement of the toxic turn of Aβ42 in the intracellular oligomer formation.

Potential of Using Vitamin E Derivatives in the Treatment of AD. We showed the protective effect of Trolox, an aqueous derivative of vitamin E, against the neurotoxicity induced by Aβ42. Osakada et al. proposed the role of α-tocotrienol in neuroprotection using cultured striatal neuron as the most potent analogue of vitamin E.[32] A clinical survey of the dietary intake of antioxidants based on over 5000 participants in The Netherlands suggested that high dietary intake of vitamin C and vitamin E may decrease the risk of AD.[33] The early supplementation of vitamin E reduced amyloid deposition in a mouse model of AD.[34] Mice deficient in the α-tocophenol transfer protein exhibited increased Aβ deposition via the impairment of Aβ clearance in AD mice.[35] According to these reports and our results, vitamin E and its derivatives could be therapeutic modalities for AD. Other in vivo studies of other vitamins revealed that several AD-like phenotypes were restored by vitamin A [36] and vitamin C treatment.[37] However, serious overdose effects of vitamin A were reported,[38] and thus, determination of the appropriate dose is required.

■ CONCLUSION

Collectively, we demonstrated the significance of the toxic conformer of Aβ42 in the neuronal damages and intracellular ROS production. The toxic turn structure at positions 22 and 23 in Aβ42 could be an attractive target for AD therapeutics such as aggregation inhibitors and antioxidants. It is noted that the increase of 11A1-reactive Aβ assembly in Aβ42 preceded Aβ42-induced neurotoxicity in primary neurons (Figures 3 and 4). The detection of the toxic conformer in E22P-Aβ42 even after dissolution (Figure 4A) did not contradict the trimer formation of Aβ42-lactam(22K-23E), in which the side chains at positions 22 and 23 are covalently linked, without incubation in Western blotting experiments.[9] Further approaches using size exclusion chromatography or surface-enhanced laser desorption ionization time-of-flight mass spectrometry would be required to identify the distribution of these oligomers under the native condition.

We recently reported that the E22Δ mutation, favoring oligomer formation,[39] promoted the β-sheet transformation,

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radical production, and synaptic dysfunction of Aβ42 in a manner similar to E22P-Aβ42.[40] Mori, Tomiyama, and colleagues reported that intraneuronal Aβ oligomers induce neuronal death through enhancement of endoplasmic reticulum stress endosomal/lysosomal leakage and mitochondrial dysfunction.[41] The relevance of this mutation to toxic turn formation in the progression of AD remains to be investigated.

METHODS

Materials. Neurobasal medium, B-27 supplement, and H_2DCFDA were purchased from Life Technologies (NY, U.S.). Sodium glutamate, L-glutamine, 1 mol/L ammonium solution, MTT, 2-propanol, SDS, and methanol were purchased from Nacalai Tesque (Kyoto, Japan). Trolox was from Cayman Chemical Company (MI, U.S.). Congo red was obtained from WAKO (Osaka, Japan). PVDF membrane (0.45 μm) was obtained from Millipore (MA, U.S.). The antibodies against Aβ1−17 (6E10) and Aβ17−24 (4G8) were purchased from Covance (CA, U.S.). The antibody against the toxic turn of Aβ22−23 (11A1) was provided by Immuno-Biological Laboratories (Gunma, Japan). ECL was from GE Healthcare Biosciences (Bucks, U.K.).

Neuronal Cultures. The animals were treated in accordance with the guidelines of the Kyoto University Animal Experimentation Committee and the guidelines of The Japanese Pharmacological Society. Neuronal cultures were obtained from the cerebral cortices of fetal Wistar rats (Nihon SLC, Shizuoka, Japan) at 17−19 days of gestation as described previously.[42,43] Cultures were maintained in Neurobasal medium with 2% B-27 supplement, 25 μM sodium glutamate, and 0.5 mM L-glutamine at 37 °C in a humidified atmosphere of 5% CO_2. After 4 days in culture, medium was replaced with sodium glutamate-free Neurobasal medium. Only mature cultures (9−10 days in vitro) were used for the experiments. In all experiments, B-27 supplement without antioxidants was utilized to examine the effect of reagents on the cellular ROS production.

MTT Assay. Neurotoxicity was assessed by MTT assay according to the previously reported protocol[44] with slight modifications. Aβ42, synthesized as previously reported,[44] was dissolved in 0.02% NH_4OH to 200 μM. After 30 min of incubation on ice, Aβ42 solution diluted by 0.02% NH_4OH to appropriate concentrations was added to the culture medium (1−20 μM). A 24 h pretreatment was performed in the Trolox treatment experiments (0.3 or 1.0 mM) but not in the Congo red treatment experiments (20 or 200 μM). When Aβ42 was added in the Trolox experiment, the medium containing Trolox was replaced with fresh medium only including Aβ42 because Trolox strongly affected the aggregative ability of Aβ42 (data not shown). After treatment at 37 °C for 8, 16, 24, 36, and 48 h, the culture medium was replaced with medium containing 0.5 mg/mL MTT, and cells were incubated for 30 min at 37 °C. 2-Propanol was added to lyse the cells, and absorbance was measured at 595 nm with an absorption spectrometer (microplate reader model 680, Bio-Rad Laboratories, CA, U.S.). The absorbance obtained by the addition of vehicle was taken as 100%. The medium of vehicle treatment of each experiment contained 0.002% NH_4OH. In the investigation of the effects of Trolox and Congo red, the medium of vehicle treatment also included 0.1% dimethyl sulfoxide.

Dot Blotting. Aβ42 was dissolved in 0.02% NH_4OH at 200 μM. After 30 min of incubation on ice, Aβ42 solution was diluted to 20 μM by 50 mM phosphate buffered saline (PBS) and incubated at 37 °C. At each time point, Aβ42 solution was gently mixed, and 2 μL of the solution was applied to a methanol-hydrophilized PVDF membrane. After 10 min, the membrane was blocked by 2.5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20. After blocking, the membrane was incubated with one of the primary antibodies, anti-Aβ1−17 (6E10, 1 μg/mL), anti-Aβ17−24 (4G8, 1 μg/mL), or 11A1 (1 μg/mL) [12] overnight at 4 °C followed by incubation with the secondary antibody for 1 h at room temperature. Development was performed with the ECL system.

Estimation of Intracellular ROS Levels. The intracellular ROS levels were examined by the DCF assay as described previously.[45] H_2DCFDA, a cell-permeable fluorescent probe, after treatment with cellular esterase, reacts with intracellular oxygen metabolites, resulting in the formation of DCF. Preincubation for 24 h was performed in the Trolox treatment experiment (1.0 mM) but not in the Congo red treatment experiment (50 or 200 μM). Similar to the MTT assay, when Aβ42 was added in the Trolox experiment, the medium containing Trolox was replaced with fresh medium containing only Aβ42. After incubation with Aβ42 (5 or 20 μM) and/or samples for 24 h, the medium was replaced with medium containing 30 μM H_2DCFDA, and cells were incubated for 30 min at 37 °C. After being washed twice with prewarmed PBS, the cells were solubilized with PBS containing 1% SDS. DCF fluorescence in the lysate was measured at 485 nm excitation and 525 nm emission by spectroscopic microplate reader (FLEX STATION II, Molecular Devices, CA, U.S.). The intrinsic fluorescence of Congo red was subtracted. The medium of vehicle treatment of each experiment contained 0.002% NH_4OH. In the investigation of the effects of Trolox and Congo red, the medium of vehicle treatment also included 0.1% dimethyl sulfoxide.

Statistics. The statistical significance of differences was analyzed by one-way analysis of variance and post hoc multiple comparisons using Tukey's test. Statistical significance was defined as $P < 0.05$. All data were expressed as the mean ± SEM.

ASSOCIATED CONTENT

Supporting Information

Figure illustrating the neurotoxicity and oligomer formation of the Arctic mutant of Aβ42 (E22G-Aβ42). This material is available free of charge via the Internet at http://pubs.acs.org.

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N.I., T.K., K.M., K.I., and A.A. designed the research. N.I., M.S., and K.M. performed the research. N.I., T.K., K.M., K.I., and A.A. analyzed data, and N.I., T.K., K.M., K.I., and A.A. wrote the paper.

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Notes

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ABBREVIATIONS

AD, Alzheimer's disease; Aβ, amyloid β-protein; Aβ40, 40-mer amyloid β-protein; Aβ42, 42-mer amyloid β-protein; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; Wt, wild type; DCF, 2′,7′-dichlorodihydrofluorescein; H_2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; PBS, phosphate-buffered saline; Veh, vehicle

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