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Amyloid β induces neuronal cell death through ROS-mediated ASK1 activation

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Abstract

Amyloid β (Aβ) is a main component of senile plaques in Alzheimer's disease and induces neuronal cell death. Reactive oxygen species (ROS), nitric oxide and endoplasmic reticulum (ER) stress have been implicated in Aβ-induced neurotoxicity. We have reported that apoptosis signal-regulating kinase 1 (ASK1) is required for ROS- and ER stress-induced JNK activation and apoptosis. Here we show the involvement of ASK1 in Aβ-induced neuronal cell death. Aβ activated ASK1 mainly through production of ROS but not through ER stress in cultured neuronal cells. Importantly, *ASK1*$^{-/-}$ neurons were defective in Aβ-induced JNK activation and cell death. These results indicate that ROS-mediated ASK1 activation is a key mechanism for Aβ-induced neurotoxicity, which plays a central role in Alzheimer's disease.

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Keywords: amyloid β; ASK1; JNK; reactive oxygen species (ROS); neuronal cell death

Abbreviations: Aβ, amyloid β; ROS, reactive oxygen species; NO, nitric oxide; ER, endoplasmic reticulum; ASK1, apoptosis signal-regulating kinase 1; AD, Alzheimer's disease; FAD, familial AD; SAD, sporadic AD; APP, amyloid precursor protein; PS, presenilin; NOSs, NO synthases; MAPKKK, mitogen-activated protein kinase kinase kinase; PG, propyl gallate; VitE, vitamin E; hROS, highly reactive oxygen species; MTT, 3′-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; GFAP, glial fibrillary acidic protein; HBSS, Hank's balanced salt solution; IP, immunoprecipitation; Tg, thapsigargin; WB, immunoblotting

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder clinically characterized by progressive loss of memory. Common pathological features of familial AD (FAD) and sporadic AD (SAD) include senile plaques, neurofibrillary tangles and neuronal loss in brain regions involved in learning and memory. A role of the accumulation of amyloid β (Aβ) peptides in forming fibrillar deposits, a principal component of senile plaques, has been suggested by several findings.[1,2] Aβ peptides are 39–43amino-acid peptides cleaved by β- and γ-secretases from the amyloid precursor protein (APP).[3] FAD has been linked to mutations in three different genes: the APP gene, presenilin (PS) 1 gene and PS2 gene. Expression of these mutant proteins in cultured cells results in increased production of fibrillar Aβ peptides. In addition, fibrillar Aβ, but not soluble Aβ, is toxic to cultured neuronal cells.[2] These findings suggest that aggregation of Aβ plays an important role in the development of AD. Thus, it is important to elucidate the molecular mechanisms of Aβ-induced neuronal cell death. Several studies have shown that dying cells display the characteristics of apoptosis in AD brains and in cultures of neurons exposed to Aβ.[4] It has been reported that Aβ impairs mitochondrial redox activity and increases the generation of reactive oxygen species (ROS).[5–7] Several studies also suggest that Aβ-induced oxidative stress leads to apoptotic neuronal cell death that can be inhibited by antioxidants.[7–9] Nitric oxide (NO) synthesized by NO synthases (NOSs) also appears to participate in the pathogenesis of AD. Pathologic studies have suggested a functional link between NO and AD, in that neurofibrillary tangles in AD brain contain inducible NOS and exhibit nitrotyrosine formation in proteins.[10,11] Induction of neurotoxicity by FAD-linked mutations of PS1 is inhibited by NOS inhibitors.[12] These findings suggest that ROS and NO may be important mediators of Aβ-induced neuronal cell death in the development of AD. However, the specific target of ROS and/or NO in AD remains to be elucidated.

Accumulation of unfolded proteins within the lumen of the endoplasmic reticulum (ER) induces ER stress, and ER stress has been implicated in neurodegenerative disorders including AD,[13] Parkinson's disease[14] and polyglutamine diseases.[15] We have recently shown that the mammalian mitogen-activated protein kinase kinase kinase (MAPKKK) termed apoptosis signal-regulating kinase 1 (ASK1) constitutes an IRE1–TRAF2–ASK1 cascade that eventually activates JNK in ER stress signaling.[15] We also demonstrated that primary

neurons derived from *ASK1*$^{-/-}$ mice were resistant to ER stress-induced cell death, and that the ASK1-mediated apoptosis pathway plays an important role in polyglutamine diseases.

Aβ induces activation of JNK and phosphorylation of c-Jun.[16,17] In addition, Aβ-induced neuronal cell death is inhibited either by the expression of dominant-negative mutant of c-Jun, by treatment with a JNK inhibitor or by the targeted disruption of c-Jun or JNK3.[16–18] Nevertheless, the mechanism of Aβ-induced JNK activation is unknown. ASK1 is activated in response to H_2O_2, TNF and ER stress through distinct mechanisms.[19,20] Overexpression of wild-type or activated mutant of ASK1 induces apoptosis in various cells through mitochondria-dependent caspase activation.[19,21,22] Recently, we showed that ROS-induced sustained activation of JNK is lost in *ASK1*$^{-/-}$ MEFs and that *ASK1*$^{-/-}$ cells are less susceptible than *ASK1*$^{+/+}$ cells to ROS.[23] These observations suggested that ASK1 is a key element in ROS-induced apoptosis.

In the present study, activation of ASK1 through ROS was found to constitute a major signaling pathway for Aβ-induced cell death, which plays an important role in the pathogenesis of AD.

Results and discussion

Aβ activates ASK1–JNK pathway

The molecular mechanism of Aβ-induced neuronal cell death is not well understood. It was previously shown that fibrillar Aβ activated JNK in cortical neurons[16,17]; however, the molecular mechanism by which Aβ activates JNK is unknown. Since ASK1 is a MAPKKK that activates the JNK signaling cascade, we examined whether ASK1 is involved in Aβ-induced JNK activation. We first examined the effect of Aβ on the catalytic activity of ASK1 by an anti-phospho-ASK1 antibody that monitors activating autophosphorylation of ASK1.[24] Treatment of PC12 cells with Aβ activated endogenous ASK1 in a dose-dependent manner (Figure 1a). Activation of ASK1 was observed within 4 h and continued until 16 h after stimulation with Aβ, in parallel with JNK activation (Figure 1b).

Aβ activates ASK1 independent of the ER stress pathway

ER stress has been suggested to be involved in the pathogenesis of AD.[13,25] Since ASK1 is required for ER stress-induced cell death,[15] Aβ might activate the ASK1–JNK pathway through ER stress. We thus examined whether Aβ induces ER stress as assessed by band-shift analysis of ER-resident transmembrane kinases, IRE1 and PERK. IRE1 and PERK were clearly activated by treatment with thapsigargin (Tg), which triggers ER stress by depletion of luminal calcium stores, but not by treatment with Aβ, in PC12 cells (Figure 2a). Importantly, Aβ and Tg activated JNK to a similar extent, suggesting that Aβ activates ASK1 and JNK without causing ER stress. Possible involvement of ER stress in Aβ-induced ASK1–JNK pathway was further assessed by using primary neurons derived from E14.5 mice. Aβ did not induce mRNAs of BiP, also known as GRP78, and CHOP, the other ER stress

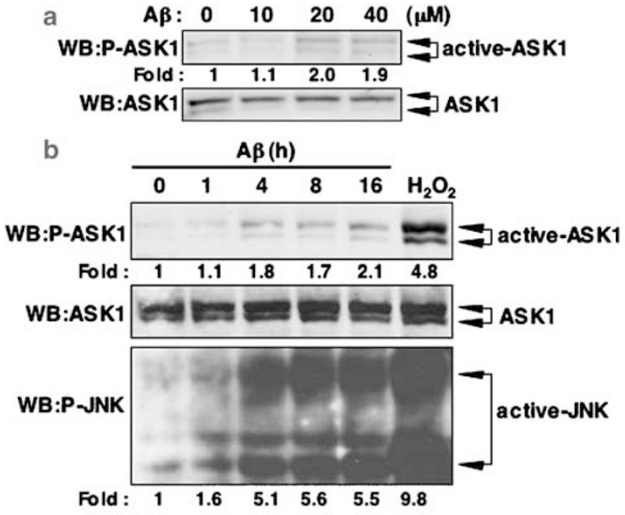

Figure 1 Aβ activates the ASK1–JNK pathway in PC12 cells. (**a**) Dose-dependent activation of ASK1 by Aβ. PC12 cells were treated with $A\beta_{1-42}$ at the indicated dose for 8 h. Cell lysates were subjected to immunoblotting (WB) with antibody to phospho-ASK1 (P-ASK1). The membrane was reprobed with antibody to ASK1 for loading controls. Fold activation of ASK1 is indicated. (**b**) Time course of Aβ-induced ASK1 and JNK activation. PC12 cells were treated with 50 μM $A\beta_{25-35}$ for the indicated time periods or with 1 mM H_2O_2 for 30 min. Cell lysates were subjected to WB with antibodies to phospho-ASK1 and phospho-JNK (P-JNK). The membrane was reprobed with antibody to ASK1. Fold activations of ASK1 and JNK are indicated

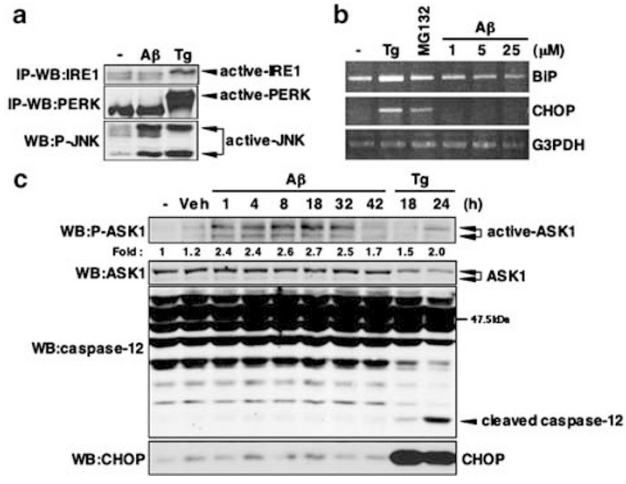

Figure 2 Aβ does not induce ER stress. (**a**) Aβ does not induce activation of endogenous IRE1 and PERK in PC12 cells. PC12 cells were treated with 100 μM $A\beta_{1-42}$ for 16 h or 20 μM Tg for 30 min. Cells were lysed and analyzed by immunoprecipitation (IP)–immunoblotting (WB) with anti-IRE1α and anti-PERK antisera. Activation of JNK was confirmed as described in Figure 1b. (**b**) Aβ does not induce mRNAs of BiP and CHOP in primary neurons. The results of RT-PCR following treatment with 2 μM Tg for 1 h, 0.1 μM MG132 for 48 h and the indicated dose of $A\beta_{25-35}$ for 6 h are shown. Expression of G3PDH was examined as a quantity control (bottom). (**c**) Aβ does not cleave caspase-12 in PC12 cells. PC12 cells were treated with 40 μM $A\beta_{1-42}$ or 2 μM Tg for the indicated time periods. Cell lysates were subjected to WB with antibody to phospho-ASK1. The membrane was reprobed with antibody to ASK1 for loading controls. Fold activation of ASK1 is indicated. Cleavage of caspase-12 was assessed using anti-caspase-12 antiserum. Induction of CHOP protein was analyzed using anti-CHOP antiserum

marker (Figure 2b). In contrast, induction of BiP and CHOP was clearly observed in these cells by treatment with Tg or MG132, a proteasome inhibitor that can trigger ER stress through proteasome dysfunction.[15] These results suggest that Aβ does not induce ER stress in neuronal cells. Although ER stress is unlikely to be involved in Aβ-induced neurotoxicity, caspase-12 might be activated by Aβ in an ER stress-independent mechanism. Since cleavage is required for ER stress-induced activation of caspase-12,[26,27] we next analyzed the kinetics of Aβ-induced activation of ASK1 and cleavage of caspase-12. Treatment of PC12 cells with 2 μM of Tg for 24 h induced activation of ASK1 and cleavage of caspase-12 (Figure 2c). Aβ-induced activation of ASK1 was also observed within 1 h; however, cleaved caspase-12 was hardly detectable after treatment with Aβ (Figure 2c). Induction of CHOP was also observed by Tg but not by Aβ (Figure 2c). These results indicate that induction of ER stress is unlikely to be required for Aβ-induced activation of the ASK1–JNK pathway.

ROS mediates Aβ-induced ASK1 activation

Several reports have shown that NO synthesized by NOS participates in the pathogenesis of AD.[10,12] Since ASK1 has been suggested to be involved in NO-induced death of PC12 cells,[28] we examined whether Aβ-induced ASK1 activation is mediated by NO. Treatment of PC12 cells with NOR1, an NO donor that can spontaneously release NO, strongly activated endogenous ASK1, whereas L-NAME (inhibitor of NO formation by NOS) had only a marginal effect on Aβ-induced ASK1 activation (Figure 3a). We next examined whether Aβ induces NO within cells by using a fluorescence probe, DAF-2 DA, that can directly detect NO.[29] Although NO was easily detected after treatment with NOR1 (Figure 3c), we could not detect apparent production of NO by treatment with Aβ (Figure 3d). These results suggest that NOS-induced synthesis of NO may not be the main mechanism for Aβ-induced ASK1 activation.

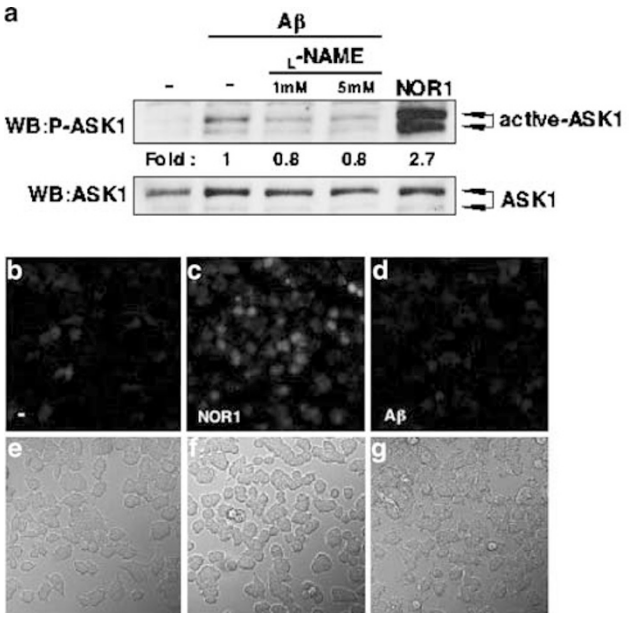

Figure 3 NO-independent activation of ASK1 by Aβ. (**a**) Marginal inhibition of Aβ-induced ASK1 activation by NOS inhibitor. PC12 cells were mixed with 1 or 5 mM L-NAME for 30 min prior to treatment with 40 μM $A\beta_{1-42}$ for 8 h. PC12 cells were treated with 0.5 mM NOR1 for 10 min. Fold activation of ASK1 is indicated. (**b–g**) Fluorescence image of NO in PC12 cells. PC12 cells were stimulated with 0.5 mM NOR1 for 10 min (**c**, **f**) or treated with 40 μM $A\beta_{1-42}$ for 8 h (**d**, **g**). Fluorescence images (**b–d**) of cells were acquired after loading with 100 nM DAF-2 DA for 30 min. Cell morphology was determined by Nomarski differential interference contrast microscopy (**e–g**)

It was reported that ROS is involved in Aβ-induced neuronal cell death.[7,8] Since ASK1 is known to be activated by ROS[19] and to be required for ROS-induced apoptosis,[23] we examined whether Aβ-induced ASK1 activation is mediated by ROS. To evaluate the effect of antioxidants on Aβ-induced ASK1 activation in PC12 cells, we used three types of antioxidants, propyl gallate (PG; ROS scavenger), MnTBAP (SOD mimetic and peroxynitrite scavenger) and vitamin E (VitE; a physiological membrane-bound antioxidant that protects cell membrane lipids from oxidative damage), each of which has been reported to protect cells from Aβ-induced neuronal cell death but by different antioxidant mechanisms.[30] Each of these anti-oxidants inhibited H_2O_2-induced ASK1 activation (Figure 4b). Aβ-induced ASK1 activation was strongly inhibited by PG and VitE, and MnTBAP also partially inhibited ASK1 activation (Figure 4a). These results suggest that ROS may be an intermediate of Aβ-induced ASK1 activation. We next examined whether Aβ induces ROS within cells by using a fluorescence probe, HPF, that can selectively detect highly reactive oxygen species (hROS) such as hydroxyl radical (•OH) and peroxynitrite ($ONOO^-$).[31] The fluorescence intensity of PC12 cells was clearly increased after treatment with H_2O_2 for 30 min (Figure 4d), and this increase was blocked by PG (Figure 4e), MnTBAP and VitE (data not shown). We could clearly visualize Aβ-induced production of ROS (Figure 4f). Fluorescence was abolished in the presence of PG, MnTBAP or VitE (Figure 4g–i). These findings indicate that Aβ induces the synthesis of ROS within cells.

ASK1 is required for Aβ-induced JNK activation and neuronal cell death

ASK1 is required for ROS-induced activation of JNK.[19,23] We assessed the requirement of ASK1 for Aβ-induced JNK activation by using a primary neuronal culture derived from E14.5 *ASK1*$^{-/-}$ mice. Activation of endogenous JNK by Aβ was lost in *ASK1*$^{-/-}$ cells (Figure 5a). Cell death was next determined by 3′-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay. Aβ-induced cell death was observed in about 80% of *ASK1*$^{+/+}$ cells (Figure 5b). *ASK1*$^{-/-}$ cells were much less sensitive to the toxic effects of Aβ than *ASK1*$^{+/+}$ cells (Figure 5b). Although these cells derived from *ASK1*$^{+/+}$ and *ASK1*$^{-/-}$ mice were mixed neuronal and glial cells, more than 90% of them were positive for microtubule-associated protein (MAP) 2 (a neuronal marker, data not shown). We may thus conclude that ASK1 is required for Aβ-induced JNK activation and neuronal cell death. On the other hand, the partial resistance of *ASK1*$^{-/-}$ cells to the Aβ-induced toxicity (Figure 5b) suggests that an ASK1-independent cell death pathway may also exist. Finally, we examined

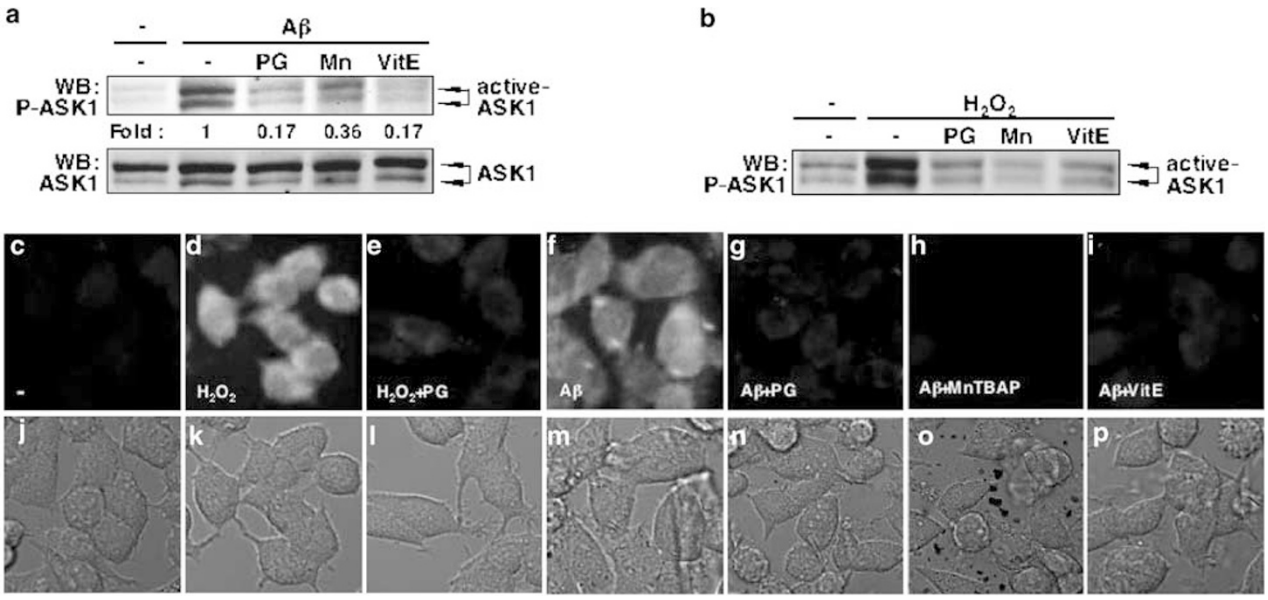

Figure 4 ROS-dependent activation of ASK1 by Aβ. (**a**, **b**) Inhibition of Aβ- and H_2O_2-induced ASK1 activation by antioxidants. PC12 cells were pretreated with antioxidants (20 μM propyl gallate: PG; 40 μM MnTBAP: Mn; 1 mg/ml vitamin E: VitE) for 30 min prior to treatment with 35 μM $A\beta_{1-42}$ for 8 h and 1 mM H_2O_2 for 30 min. Immunoblotting was performed as described in Figure 1a. Fold activation of ASK1 is indicated. (**c–p**) Fluorescence image of ROS in PC12 cells treated with Aβ. PC12 cells were stimulated with 1 mM H_2O_2 for 30 min without (**d**, **k**) or with PG (**e**, **l**), or treated with 40 μM $A\beta_{1-42}$ for 8 h without (**f**, **m**) or with PG (**g**, **n**), MnTBAP (**h**, **o**) or VitE (**i**, **p**). Fluorescence images (**c–i**) of cells were acquired after loading with 100 nM HPF for 30 min. Cell morphology was determined by Nomarski differential interference contrast microscopy (**j–p**). This experiment was performed three times with similar results

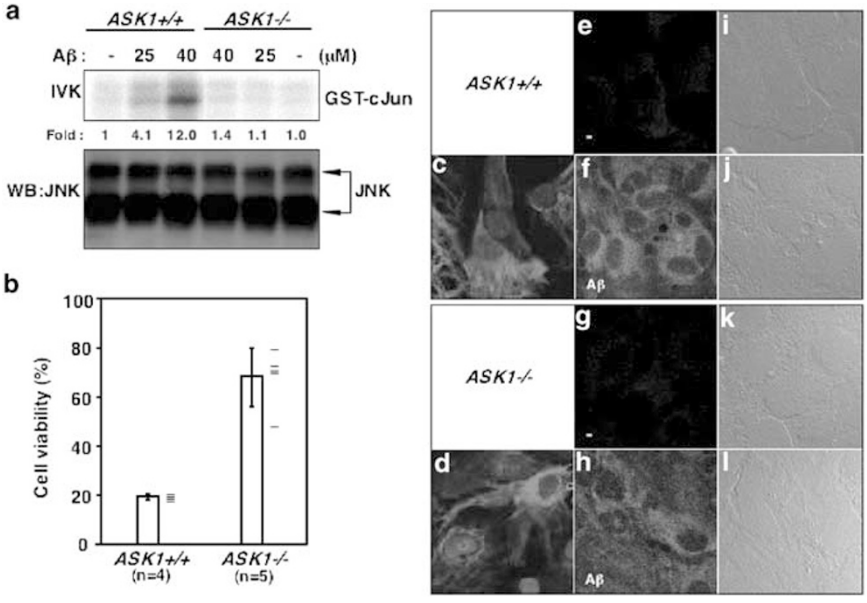

Figure 5 Requirement of ASK1 for Aβ-induced JNK activation and cell death. (**a**) Lack of Aβ-induced JNK activation in $ASK1^{-/-}$ neuronal cells. $ASK1^{+/+}$ and $ASK1^{-/-}$ cells were treated with or without 25 or 40 μM $A\beta_{1-42}$ for 16 h. JNK was immunoprecipitated by anti-JNK antibody and subjected to immune complex kinase assay as described in Materials and Methods. (Top) *In vitro* kinase assay (IVK) for JNK activity. Expression of JNK (bottom) in the same lysate is shown. Fold activation of JNK is indicated. (**b**) Lack of Aβ-induced cell death in $ASK1^{-/-}$ cells. $ASK1^{+/+}$ and $ASK1^{-/-}$ primary cultured neuronal cells were treated with 25 μM $A\beta_{25-35}$ for 3 days. The graph shows cell viability determined by MTT assay as described in Materials and Methods. Data are means (±S.E.) of four independent experiments in $ASK1^{+/+}$ and five independent experiments in $ASK1^{-/-}$ derived from independent embryos. (**c–l**) Fluorescence images of ROS in $ASK1^{+/+}$ and $ASK1^{-/-}$ astrocytes treated with Aβ. $ASK1^{+/+}$ (**c**) and $ASK1^{-/-}$ (**d**) astrocytes were stained with anti-GFAP antibody and Hoechst 33258. $ASK1^{+/+}$ (**e**, **f**, **i**, **j**) and $ASK1^{-/-}$ (**g**, **h**, **k**, **l**) primary astrocytes were treated without (**e**, **i**, **g**, **k**) or with 40 μM $A\beta_{1-42}$ for 4 h (**f**, **j**, **h**, **l**). Fluorescence images (**e–h**) of cells were acquired as described in Figure 4. Cell morphology was determined by Nomarski differential interference contrast microscopy (**i–l**). The experiment was performed three times with similar results

the effect of ASK1 deficiency on Aβ-induced production of ROS. Due to the nonspecific high background of ROS in the primary neuronal culture, we examined Aβ-induced production of ROS in *ASK1*$^{+/+}$ and *ASK1*$^{-/-}$ primary astrocytes, which were positive for glial fibrillary acidic protein (GFAP) (Figure 5c and d). Aβ-induced production of ROS was indistinguishable between *ASK1*$^{+/+}$ and *ASK1*$^{-/-}$ astrocytes (Figure 5f and h). These results suggest that synthesis of ROS by Aβ does not require ASK1.

The accumulation of Aβ peptides to form fibrillar deposits is closely related to the loss of neuronal cells in AD. Pathologic and biochemical studies suggest that ROS synthesized by fibrillar Aβ has neurotoxic effects.[32,33] Recent studies suggested that monomeric (soluble) Aβ acts as a natural antioxidant that prevents neuronal cell death caused by oxidative stress, whereas fibrillar Aβ is an ROS generator and neurotoxic.[34] Gly33 and Met35 of $A\beta_{1-42}$ peptide have been reported to be essential for ROS production and neurotoxicity.[35] However, the precise molecular mechanism by which Aβ leads to neuronal cell death has not been elucidated. In this study, we have shown for the first time that Aβ targets the ASK1–JNK proapoptotic pathway through ROS production. Since the molecular mechanism of Aβ-induced production of ROS has not been elucidated, the identification of the target molecules of Aβ for the synthesis of ROS should shed light on the regulation of Aβ-induced neuronal cell death.

In conclusion, the results presented here strongly suggest that ROS-induced ASK1 activation by Aβ is an important step in the pathogenesis of AD. ASK1 may thus be a therapeutic target for prevention and treatment of AD.

Materials and Methods

Cell cultures

PC12 cells and primary neurons were maintained as described.[15] For primary astroglial cultures, telencepharons from neonatal C57BL/6 mice were minced into small pieces with a scalpel in Hank's balanced salt solution (HBSS), and treated with HBSS containing 0.25% trypsin and 0.1% DNase I for 15 min at 37°C. Dissociated cells were cultured in MEM with Earle's supplemented with 10% FBS and penicillin G (100 U/ml) containing 30 mM glucose. The purity of astrocytes was assessed by immunofluorescent staining for anti-GFAP antibody (DAKO).

Reagents

$A\beta_{25-35}$ and $A\beta_{1-42}$ were purchased from Bachem and American Peptide Company, respectively. $A\beta_{25-35}$ was dissolved in water at 1 mM and $A\beta_{1-42}$ was dissolved in water containing 0.1% NH_3 at 1 mM. Dissolved Aβ peptides were mixed with the same volume of PBS and incubated at 37°C for 3–5 days before use. PG, MnTBAP and VitE were purchased from Calbiochem.

Western blot analysis

Cells were lysed on ice in a lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM EDTA (pH 7.5), 1% Triton X-100 and 1% deoxycholate, and cell extracts were clarified by centrifugation, resolved on SDS–PAGE and transferred onto PVDF membranes. After blocking with 5% skim milk in TBS-T (50 mM Tris-HCl (pH 8.0), 150 mM NaCl and 0.05% Tween 20), the membranes were probed with antibodies to ASK1, phospho-ASK1, JNK, phospho-JNK, CHOP and caspase-12. Blots were developed with ECL (Amersham). The amount of protein was quantified by NIH Image.

RT-PCR, immunoblotting and band-shift analysis for IRE1 and PERK

RT-PCR, immunoblotting and band-shift analysis for IRE1 and PERK have been described.[15]

Bioimaging of NO and ROS

PC12 cells and astrocytes were seeded onto a glass-bottomed dish. For detection of NO, the cells were mixed with 100 nM DAF-2 DA[29] for 1 h after treatment with Aβ for 8 h or before treatment with 0.5 mM NOR1 for 10 min. Fluorescence images were acquired after washing with medium. For the detection of ROS, the cells were mixed with 100 nM HPF after incubation with Aβ for 8 h or with H_2O_2 for 30 min in the presence or absence of antioxidants. After 30 min of incubation at 37°C, fluorescence images were acquired using an LSM510 confocal laser scanning unit coupled to an Axiovert 100M inverted microscope with a C Apochromat $\times$ 40/1.2 objective lens (Carl Zeiss). The excitation wavelength was 488 nm, and the emission was filtered using a 505–530 nm barrier filter.

Immune complex kinase assay for JNK

Primary neurons (3×10^6) in six-well plates were lysed with the lysis buffer and immunoprecipitated with anti-JNK polyclonal antibody (Santa Cruz). The kinase assay using GST-cJun (1–79) has been described.[20] The amount of JNK protein was determined by immunoblotting with anti-JNK polyclonal antibody.

MTT assay

Viability of primary neurons was determined as described.[15] The relative number of surviving cells was determined in triplicate using the value for cells stimulated with vehicles as 100%.

Acknowledgements

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