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Characterization of the Toxic Mechanism Triggered by Alzheimer's Amyloid-β Peptides Via p75 Neurotrophin Receptor in Neuronal Hybrid Cells

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Neuronal pathology of the brain with Alzheimer's disease (AD) is characterized by numerous depositions of amyloid-β peptides (Aβ). Aβ binding to the 75-kDa neurotrophin receptor (p75NTR) causes neuronal cell death. Here we report that Aβ causes cell death in neuronal hybrid cells transfected with p75NTR, but not in non-transfected cells, and that $p75NTR^{L401K}$ cannot mediate Aβ neurotoxicity. We analyzed the cytotoxic pathway by transfecting pertussis toxin (PTX)-resistant G protein α subunits in the presence of PTX and identified that $G\alpha_o$, but not $G\alpha_i$, proteins are involved in p75NTR-mediated Aβ neurotoxicity. Further investigation suggested that Aβ neurotoxicity via p75NTR involved JNK, NADPH oxidase, and caspases-9/3 and was inhibited by activity-dependent neurotrophic factor, insulin-like growth factor-I, basic fibroblast growth factor, and Humanin, as observed in primary neuron cultures. Understanding the Aβ neurotoxic mechanism would contribute significantly to the development of anti-AD therapies.

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Key words: L401K mutant; G_o; JNK; NADPH oxidase; caspase

Alzheimer's disease (AD) is pathologically characterized by extensive neuronal loss, intracellular neurofibrillary tangles, and extracellular senile plaques. The major constituent of the senile plaques is amyloid-β peptides (Aβ). Aβ is the 39–43-amino-acid peptide produced by the cleavage of a larger transmembrane protein, termed *amyloid precursor protein* (AβPP). The formation and accumulation of Aβ are inferred to be implicated in the pathogenesis of AD. Although Aβ treatment causes massive cell death in cultures of primary neurons as well as in neuronal cell lines, the underlying mechanism is not fully understood.

With regard to the mechanism for Aβ neurotoxicity, it is most important to clarify whether Aβ causes neurotoxicity by binding to cell surface receptors and, if so, what receptor molecule mediates Aβ neurotoxicity. Multiple proteins have thus far been proposed as candidates for the Aβ receptor. They are the receptor for advanced glycation end products (Yan et al., 1997b), the endoplasmic reticulum Aβ-binding dehydrogenase (ERAB; Yan et al., 1997a), the α7 nicotinic acetylcholine receptor (Wang et al., 2000), the formyl peptide receptor like-1 (Le et al., 2001), the amyloid β-binding protein-1 (BBP-1; Kajkowski et al., 2001), AβPP (Lorenzo et al., 2000), and the 75-kDa neurotrophin receptor (p75NTR; Yaar et al., 1997). Among these, ERAB, BBP-1, AβPP, and p75NTR are able to cause neuronal cell death. We directed our attention to p75NTR. The first linkage between Aβ neurotoxicity and p75NTR was noted by Rabizadeh et al. (1994), who found that expression of p75NTR enhances Aβ toxicity in PC12 cells and that nerve growth factor (NGF) binding to p75NTR inhibits Aβ toxicity. Yaar et al. (1997) found that Aβ directly binds to p75NTR and causes c-Jun N-terminal kinase (JNK) activation and apoptotic cell death in p75NTR-expressing cells. Kuner and Hertel (1998) confirmed the Aβ binding to p75NTR, activating nuclear factor-κB (NFκB) in neuroblastoma cells. Perini et al. (2002) specified the death domain (Ser^{337}-Ser^{416}) of p75NTR as the region responsible for the mediation of Aβ neurotoxicity.

p75NTR is a single-transmembrane protein with a structure similar to the tumor necrosis factor receptor, Fas, and CD40. The receptors belonging to this group have a conserved cysteine-rich domain in the extracellular region

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and the death domain in the intracellular region (Kuner and Hertel, 1998). Because NGF binding to p75NTR induces neuronal apoptosis (Kong et al., 1999; Wang et al., 2001), p75NTR is qualified as a strong candidate for the Aβ neurotoxicity receptor. On the other hand, the most recent evidence indicates that Aβ neurotoxicity is mediated by pertussis toxin (PTX)-sensitive heterotrimeric G proteins (Wei et al., 2002). Because no evidence has thus far indicated that p75NTR utilizes heterotrimeric G proteins, we investigated their involvement in p75-mediated Aβ toxicity.

In fact, there is a possibility that p75NTR can cause neuronal apoptosis via PTX-sensitive G proteins, insofar as 1) the death domain of p75NTR contains a region (Ala^{391}-Arg^{410}) structurally similar to mastoparan (Feinstein and Larhmmer, 1990; Liepinsh et al., 1997), which can directly activate PTX-sensitive $G_{i/o}$ proteins; 2) the Arg^{384}-Arg^{410} region in the intracellular domain satisfies the modified criteria for peptide sequences to activate PTX-sensitive $G_{i/o}$ proteins (Orcel et al., 2000); 3) $G_{i/o}$ proteins can mediate neuronal apoptosis via their Gβγ subunit (Giambarella et al., 1997; Hashimoto et al., 2000b); 4) neuronal cell death by AD-linked mutants of AβPP and presenilin-2 (PS2) and by antibody-bound wild-type AβPP is mediated by G_o (Hashimoto et al., 2000a, 2002a,b, 2003); and 5) G_o is the upstream mediator of JNK in AβPP-induced neurotoxicity (Hashimoto et al., 2003), and it has been shown that Aβ binding to p75NTR causes JNK activation (Yaar et al., 1997). Consistently with this notion, Rabizadeh et al. (2000) showed that the synthetic peptide corresponding to the mastoparan-like sequence (residues 364–377 of mouse p75NTR, which are identical to residues 396–409 of human p75NTR; the numbering of human p75NTR is employed here) can induce cellular apoptosis. Medina et al. (2002) found that the substitution of Lys for Leu^{401} (L401K) abolishes the proapoptotic activity of this synthetic peptide. Nevertheless, it has not been examined whether p75NTR with L401K ($p75NTR^{L401K}$) is deficient in mediating neurotoxicity.

The present study was conducted to confirm the mediation by p75NTR of Aβ neurotoxicity in neuronal hybrid cells and, if this is so, to analyze further the Aβ/p75NTR-triggered neurotoxic mechanism and to compare this with the characteristics of Aβ neurotoxicity so far reported. The results revealed that 1) p75NTR does mediate Aβ neurotoxicity; 2) $p75NTR^{L401K}$ loses this function; and 3) p75NTR-mediated Aβ neurotoxicity most likely involves G_o, JNK, NADPH oxidase, and caspases-9/3. We also found that p75NTR-mediated Aβ neurotoxicity in neuronal hybrid cells was suppressed by activity-dependent neurotrophic factor (ADNF; Brenneman and Gozes, 1996), insulin-like growth factor-I (IGF-I; Dore et al., 1997), basic fibroblast growth factor (bFGF; Mark et al., 1997), and Humanin (HN; Hashimoto et al., 2001a), as has been observed for Aβ neurotoxicity in primary neuron cultures (Hashimoto et al., 2001c; for review see Kawasumi et al., 2003). These data should contribute to the understanding of the molecular mechanism for Aβ neurotoxicity and provide essential clues leading to the development of new and effective anti-AD therapies.

MATERIALS AND METHODS

Genes and Materials

The cDNAs encoding PTX-resistant G protein α subunits (PTX-rGα: PTX-rGα_o, PTX-rGα_{i1}, PTX-rGα_{i2}, PTX-rGα_{i3}; Taussig et al., 1992) were kindly provided by Drs. R. Taussig (University of Michigan) and T. Kozasa (University of Texas Southwestern Medical Center). Mouse wild-type (wt) AβPP in pcDNA was described previously (Yamatsuji et al., 1996). Human wt AβPP cDNA in pCAG was kindly provided by Dr. F. Oyama (RIKEN, Saitama, Japan). In this study, wt AβPP was simply termed AβPP. NG-monomethyl-L-arginine methyl ester hydrochloride (L-NMMA), SP600125, SB203580, PD98059, and PTX were from Calbiochem (San Diego, CA). Glutathione ethyl ester (GEE), apocynin (4-hydroxy-3 methoxyacetophenone), diphenyleneiodonium chloride (DPI), and oxypurinol were from Sigma (St Louis, MO). Ac-DEVD-CHO (acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartyl-al), Ac-YVAD-CHO (acetyl-L-tyrosyl-L-valyl-L-alanyl-L-aspart-1-al), Ac-IETD-CHO (acetyl-L-isoleucyl-L-glutamyl-L-threonyl-L-aspart-1-al), Ac-LEHD-CHO (acetyl-L-leucyl-L-glutamyl-L-histidyl-L-aspart-1-al), Aβ1–43, Aβ1–42, Aβ1–40, Aβ25–35, and HN were from the Peptide Institute (Osaka, Japan). Aβ42–1 and Aβ40–1 were purchased from Bachem (Bendendorf, Switzerland). ADNF9 (SALLRSIPA) was chemically synthesized and used as ADNF.

Plasmid Construction

p75NTR cDNA was kindly provided by Dr. M.V. Chao (New York University School of Medicine) and was subcloned into the pECE plasmid. The EcoRI and KpnI restriction sites were added to the p75NTR cDNA by polymerase chain reaction (PCR) using KOD polymerase (Toyobo). The sense and antisense primers used were 5′-TTTTTTGAATTCACCATGGGGGCAGGTGCCACC-3′ and 5′-TTTTTTGGTACCCACCGGGGATGTGGCAGTGG-3′, respectively. The PCR product was then subcloned into pFLAG-CMV5 mammalian expression vector. Because pFLAG was a FLAG-tagged vector, the expressed p75NTR was with a FLAG tag. Unless otherwise specified, p75NTR stands for wild-type p75NTR.

$p75NTR^{L401K}$ Construction

$p75NTR^{L401K}$ was constructed using the Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA), with subsequent sequence confirmation. The sense and antisense primers used for the construction of L401K mutation were 5′-CAGCGCCACACTGGACGCCAAACGGCCGCCCTGCGCCGC-3′ and 5′-GCGGCGCAGGGCGGCCAGTTTGGCGTCCAGTGTGGCGCTG-3′, respectively.

Cells and Transfection

F11 cells were grown in Ham's F-12 plus 18% fetal bovine serum (FBS) and antibiotics. F11 cells are the hybrid of a rat embryonic day 13 primary cultured neuron with a mouse neu-

roblastoma NTG18 and have so far been used as the best model for primary neurons (Yamatsuji et al., 1996; Storms and Rutishauser, 1998; Huang et al., 2000; Sudo et al., 2000, 2001; Hagiwara et al., 2000; Ghil et al., 2000; Hashimoto et al., 2000a,b, 2001a–c, 2003; Niikura et al., 2002). For transient transfection, F11 cells were seeded at 7×10^4 cells/well in a six-well plate and cultured in Ham's F-12 plus 18% FBS for 12–16 hr. Cells were transfected with pECE or pFLAG plasmids (plasmid 1 μg, Lipofectamine 2 μl, Plus Reagent 4 μl/wells) in the absence of serum for 3 hr. After subsequent incubation with Ham's F-12 plus 18% FBS for 2 hr, cells were cultured in Ham's F-12 plus 10% FBS.

For the Aβ/AβPP experiment, F11 cells were similarly transfected with or without mouse or human full-length $A\beta PP_{695}$ cDNA. Twenty-four hours after transfection, cells were treated with or without Aβ1–42, Aβ1–40, Aβ25–35, Aβ42–1, or Aβ40–1 (the Aβ powder was directly dissolved in the medium, immediately before experiments) in serum-free Ham's F-12 with or without N2 supplement. Cell mortality was measured by trypan blue exclusion assay 48 hr after the treatment with Aβ peptides. The trypan blue exclusion assay was performed as described previously (Hashimoto et al., 2003).

Immunoblot Analysis

Immunoblot analysis of expressed p75NTR constructs was performed as follows. Cell lysates (20 μg/lane) were submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and separated proteins were transferred onto PVDF sheets. After being blocked with 10% skim milk in PBST (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4 containing 0.1% Tween 20), the blots were probed with the primary antibody, M2 anti-FLAG antibody (1/5,000; Sigma), and the secondary antibody [1/5,000 dilution, anti-mouse IgG conjugated with horseradish peroxidase (HRP; Bio-Rad, Hercules, CA)], followed by visualization of the immunoreactive bands by ECL (Amersham Pharmacia Biotech, Arlington Heights, IL).

Immunoblot analysis of expressed PTX-rGα constructs with endogenous G proteins was performed as follows. Cell lysates (20 μg/lane) were submitted to SDS-PAGE, and separated proteins were transferred onto PVDF sheets. After being blocked with 10% skim milk in PBST, the blots were probed with the primary antibody, anti-$G\alpha_{i1}$ and $G\alpha_{i2}$ antibody, and anti-$G\alpha_{i3}$ and $G\alpha_o$ antibody mixture (1/500 dilution; Calbiochem), and the secondary antibody [1/5,000 diluted anti-rabbit IgG conjugated with HRP (Bio-Rad)], followed by visualization of the immunoreactive bands by ECL.

RESULTS

Cell Death by Aβ Peptides in p75NTR-Expressing Neuronal Hybrid Cells

To examine whether Aβ induces cell death in F11 neuronal hybrid cells expressing p75NTR, we transfected F11 cells with p75NTR cDNA and treated them with various concentrations of Aβ1–43, Aβ1–42, and Aβ25–35. When F11 cells were transfected with p75NTR cDNA and treated with 25 μM Aβ1–43, the percentage of dead cells increased to 65%, whereas cell mortality was only 10% when F11 cells without transfection were treated with the same concentration, 25 μM, of Aβ1–43. The cell mortality of p75NTR-transfected F11 cells was augmented in a manner dependent on the Aβ1–43 concentrations and reached saturation by 5 μM Aβ1–43 (Fig. 1A). Aβ42–1 and Aβ40–1—the control peptides with the reverse amino acid sequences of Aβ—had no effect on p75NTR-transfected F11 cells (Fig. 1A). The dose-response curves for neuronal cell death were virtually identical among Aβ1–43, Aβ1–42, and Aβ1–40 (Fig. 1A–C). Only Aβ25–35 required a lower concentration (500 nM) for the saturated neurotoxicity (Fig. 1D). These results confirmed that p75NTR can mediate Aβ neurotoxicity in F11 neurohybrid cells.

Aβ1–42 Does Not Exert Neurotoxicity Via AβPP

Lorenzo et al. (2000) reported that Aβ peptides bind to neuronal cell surface AβPP and that AβPP gene-deficient neurons are less vulnerable to Aβ toxicity, so there is a possibility that AβPP may function as a direct receptor for Aβ neurotoxicity. We thus examined, under the same condition as used for p75NTR, whether Aβ1–42 enhances cell death in F11 cells overexpressing AβPP.

Transfection of F11 cells with mouse AβPP resulted in overexpression of the cognate holoprotein (data not shown). Although overexpression of mouse AβPP caused 30–40% levels of cytotoxicity in the absence of N2 supplement (Fig. 1E), this toxicity was attributed to simple overexpression of the extreme C-terminal Met^{677}-Asn^{695} of the cytoplasmic domain of AβPP, as our earlier study had clarified (Hashimoto et al., 2000a). We noted that this toxicity was inhibited by N2 supplement (Fig. 1E). Treatment with 25 μM Aβ1–42 did not enhance toxicity in cells overexpressing mouse AβPP in either the presence or the absence of N2 supplement (Fig. 1E). Under the same conditions, treatment of mouse AβPP-overexpressing cells with 2 μg/ml anti-AβPP antibody 22C11 resulted in 50–60% cell mortality, as reported previously (Hashimoto et al., 2003).

This was also the case with human AβPP. Under the same conditions, overexpressed human AβPP caused neurotoxicity at a level similar to that with mouse AβPP in the absence of N2 supplement (Fig. 1E). N2 supplement suppressed this basal toxicity by overexpression of human AβPP. In the presence or absence of N2 supplement, 25 μM Aβ1–42 did not enhance death of cells expressing human AβPP. Insofar as 25 μM is the concentration at which Aβ1–42/43 causes massive cell death in primary neurons (see, e.g., Hashimoto et al., 2001a–c) and forms aggregates at 37°C for 3 days (Hashimoto et al., 2001a), these data indicate that AβPP does not mediate toxicity of Aβ1–42.

Function of $p75NTR^{L401K}$

We constructed a mutant of p75NTR cDNA in which Leu^{401} in the mastoparan-like sequence (see the introductory paragraphs) had been changed to Lys ($p75NTR^{L401K}$). This substitution has been reported to

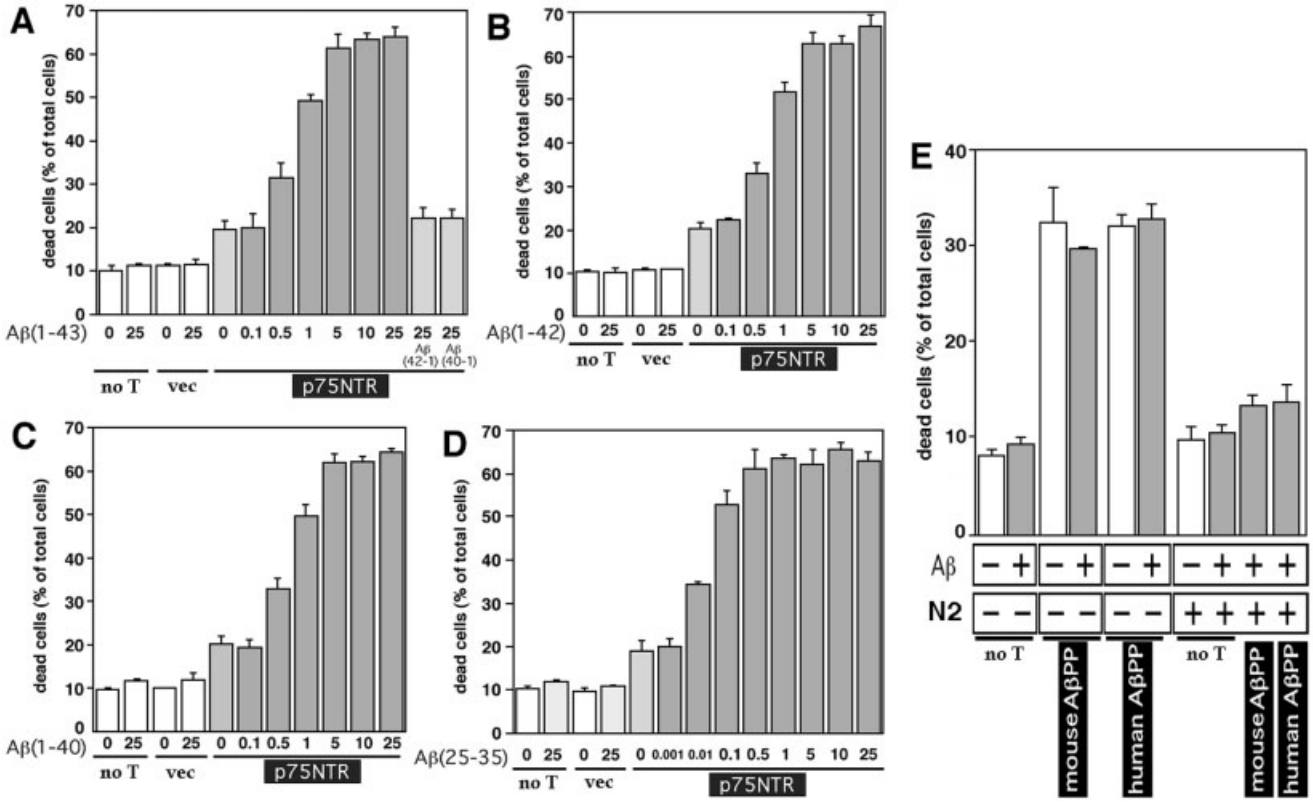

Fig. 1. Cell death by Aβ1–43, Aβ1–42, Aβ1–40, and Aβ25–35 via p75NTR but not wt AβPP. Cell death of p75NTR-transfected F11 neurohybrid cells treated with Aβ1–43 (**A**), Aβ1–42 (**B**), Aβ1–40 (**C**), and Aβ25–35 (**D**). We transfected F11 cells with p75NTR cDNA (p75NTR) and cultured them with increasing concentrations of Aβ1–43, Aβ1–42, and Aβ1–40 (100 nM, 500 nM, 1 μM, 5 μM, 10 μM, and 25 μM; A–C). In the experiment shown in D, p75NTR-transfected F11 cells were treated with Aβ25–35 (1 nM, 10 nM, 100 nM, 500 nM, 1 μM, 5 μM, 10 μM, and 25 μM). In the experiment shown in A, 25 μM Aβ42–1 and Aβ40–1 were used as negative controls. In the experiment shown in **E**, F11 cells transfected with mouse or human AβPP cDNA were treated with 25 μM Aβ1–42 in the presence or absence of N2 supplement. Aβ was added to cell culture 24 hr after transfection. Cell mortality was measured by trypan blue exclusion assay 48 hr after transfection. "No T" and "vec" indicate no transfection and empty vector transfection, respectively. Values in all figures in this paper are mean ± SD of three independent experiments.

abolish the proapoptotic function of the synthetic peptide corresponding to Ala^{396}-Gln^{409} of human p75NTR (Medina et al., 2002). No study had so far investigated whether this substitution abolishes the toxic function of intact p75NTR, so we examined $p75NTR^{L401K}$. F11 neurohybrid cells were transfected with $p75NTR^{L401K}$ cDNA and then cultured with increasing concentrations of Aβ1–42 or Aβ42–1. Cell mortality was measured 48 hr after the onset of Aβ treatment. Figure 2A shows the result, which revealed that, in $p75NTR^{L401K}$-transfected F11 neurohybrid cells, Aβ1–42 treatment was unable to enhance cell mortality, whose levels were similar to cell mortality of $p75NTR^{L401K}$-transfected cells treated with 10 μM Aβ42–1 and cell mortality of wt p75NTR-transfected cells without Aβ treatment (Fig. 2A). Under the same conditions, wt p75NTR-transfected neurohybrid cells again showed Aβ dose-dependent cell death. In these experiments, expression of $p75NTR^{L401K}$ and wt p75NTR was similar and was not affected by Aβ treatment (Fig. 2B). These data indicate that the L401K mutation nullifies the neurotoxic function of p75NTR.

PTX Inhibition of Aβ/p75NTR Induced Neurohybrid Cell Death and Involved Gα

PTX is the specific inhibitor of heterotrimeric $G_{i/o}$ proteins. We next investigated whether PTX inhibits Aβ-induced cell death in p75NTR-expressing F11 neurohybrid cells. It should be emphasized here that most recent evidence indicates that Aβ neurotoxicity is inhibited by PTX, suggesting that Aβ utilizes PTX-sensitive $G_{i/o}$ proteins to exert neurotoxicity (Wei et al., 2002). On the other hand, no evidence had thus far been obtained that p75NTR utilizes heterotrimeric G proteins for its signal transduction. Therefore, we investigated G protein involvement in p75NTR-mediated Aβ neurotoxicity.

When F11 neurohybrid cells were transfected with p75NTR cDNA and treated with 1 μg/ml PTX, 10 μM Aβ1–42 could not enhance cell mortality at all (Fig. 3A).

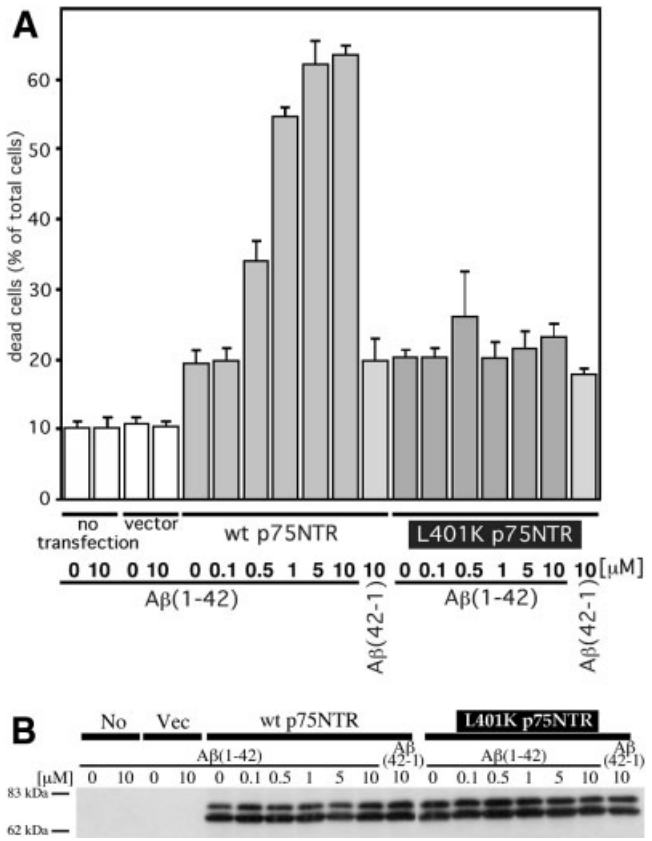

Fig. 2. Effect of L401K mutation of p75NTR on F11 neurohybrid cell death. **A**: F11 neurohybrid cells were transfected with p75NTR cDNA (wt p75NTR) or p75NTR^{L401K} cDNA (L401K p75NTR) and cultured with increasing concentrations of Aβ1–42 (100 nM, 500 nM, 1 μM, 5 μM, and 10 μM) and 10 μM Aβ42–1. Cell mortality was measured by trypan blue exclusion assay 48 hr after transfection. **B**: Expression of wt p75NTR and p75NTR^{L401K}. Expression of these proteins in the experiments shown in A was examined by immunoblotting using M2 anti-FLAG antibody.

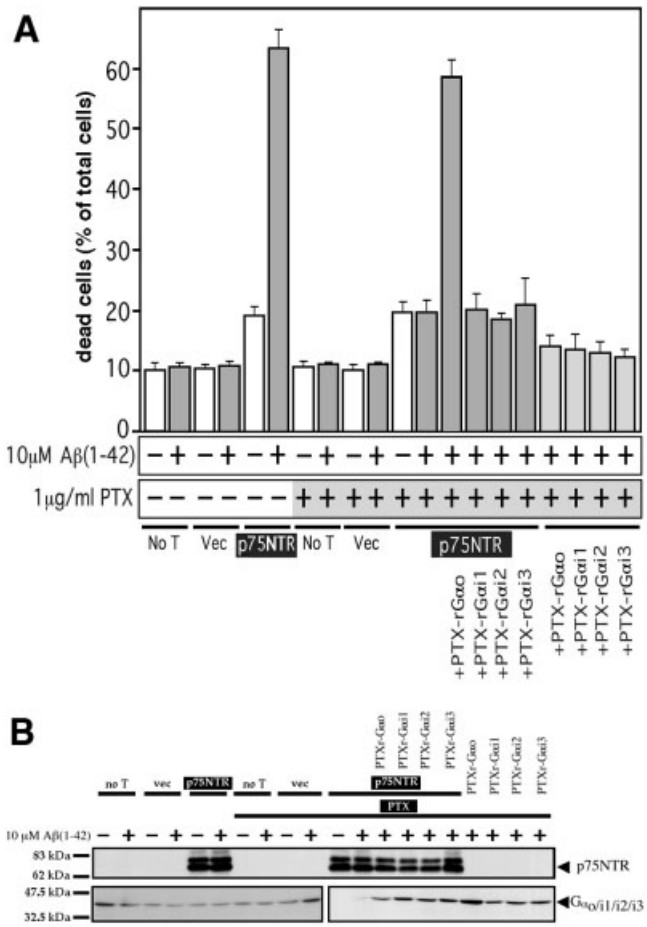

Fig. 3. Effect of PTX on Aβ 1–42/p75NTR-induced cell death and identification of PTX-sensitive G proteins involved in the mechanism. **A**: F11 cells were transfected with or without p75NTR cDNA (p75NTR) with or without PTX-resistant G protein α subunit constructs (PTX-rGα_o, PTX-rGα_{i1}, PTX-rGα_{i2}, and PTX-rGα_{i3}) and treated with or without 10 μM Aβ1–42 in the presence or absence of 1 μg/ml PTX. Aβ and PTX were added to the cell culture 24 hr after transfection. Cell mortality was measured by trypan blue exclusion assay 48 hr after transfection. "No T" and "vec" indicate no transfection case and empty vector transfection case, respectively. **B**: Expression of p75NTR in the experiments shown in A was examined by immunoblot analysis using M2 anti-FLAG antibody (upper panel). Also, expression of G$\alpha_{o/i1/i2/i3}$ was examined, as described in Materials and Methods (lower panels). Expression of endogenous G$\alpha_{o/i1/i2/i3}$ was examined in the left lower panel, which represents a picture of long exposure. Expression of transfected PTXr G$\alpha_{o/i1/i2/i3}$ was examined in the right lower panel, which represents a picture of short exposure.

In contrast, ~10% cell mortality induced by p75NTR transfection alone was not affected by PTX. p75NTR expression was not inhibited by PTX (Fig. 3B). These results indicate that p75NTR-mediated Aβ neurotoxicity was very sensitive to PTX.

We next tried to identify which of the heterotrimeric $G_{i/o}$ proteins is involved in Aβ-stimulated p75NTR-mediated neuronal death. For this purpose, we employed PTX-resistant mutant α subunits of $G_{i/o}$ proteins (Taussig et al., 1992), which can mediate coupled receptor signals even in the presence of PTX because of their Ser mutation at the Cys residue fourth from the extreme C terminus. When F11 neurohybrid cells were transfected with p75NTR and PTX-resistant Gα_o cDNA, treatment with 10 μM Aβ1–42 induced drastic cell death even in the presence of PTX (Fig. 3A). Under this condition, the level of cell mortality induced by 10 μM Aβ1–42 was almost equivalent to that induced by 10 μM Aβ1–42 in p75NTR-transfected cells in the absence of PTX. In clear contrast, in F11 cells transfected with p75NTR and any PTX-resistant Gα_{i1} cDNA, PTX-resistant Gα_{i2} cDNA, or PTX-resistant Gα_{i3} cDNA, 10 μM Aβ1–42 did not augment cell mortality in the presence of PTX (Fig. 3A). Transfection with any of these PTX-resistant Gα_o/Gα_{i1}/Gα_{i2}/Gα_{i3} cDNAs alone was not able to augment cell mortality in F11 neurohybrid cells (Fig. 3A). In these experiments, expression of p75NTR

and expression of PTX-resistant $G\alpha_o/G\alpha_{i1}/G\alpha_{i2}/G\alpha_{i3}$ proteins were similar, as shown in Figure 3B. We have confirmed that the PTX-resistant $G\alpha_{i1}/G\alpha_{i2}/G\alpha_{i3}$ cDNAs function (Hashimoto et al., 2000b). These data indicate that neuronal cell death induced by Aβ1–42 and p75NTR is specifically mediated by the PTX-sensitive G protein G_o.

Characterization of the Aβ/p75NTR-Induced Toxic Mechanism

To characterize the Aβ/p75NTR-induced toxic mechanism, we transfected F11 cells with p75NTR and cultured them with 10 μM Aβ1–42 in the presence or absence of various pharmacological inhibitors, including antioxidants, caspase inhibitors, and mitogen-activated protein kinase (MAPK) family inhibitors. First, F11 neurohybrid cells were transfected with p75NTR cDNA and cultured with 10 μM Aβ1–42 in the presence of GEE, which is an established cell-permeable antioxidant. Under this condition, cell death induced by Aβ1–42 was drastically inhibited (Fig. 4A), suggesting that the Aβ-induced neurotoxic pathway involved reactive oxygen species (ROS) production. To specify the underlying source enzyme of ROS production, we performed a pharmacological examination using the following specific inhibitors: L-NMMA, the nitric oxide synthase (NOS) inhibitor; oxypurinol, the xanthine oxidase (XO) inhibitor; and apocynin and DPI, both specific inhibitors of NADPH oxidase. Figure 4A indicates that neither L-NMMA nor oxypurinol inhibited Aβ-induced cell death. In contrast, Aβ-induced cell death was greatly suppressed by apocynin and DPI. In these experiments, L-NMMA, oxypurinol, apocynin, and DPI did not inhibit expression of p75NTR (Fig. 4B). These data suggest that the GEE target in the Aβ neurotoxic mechanism was most likely NADPH oxidase, but not NOS or XO. Consistently with these results, subunits of NADPH oxidase are present in F11 neurohybrid cells (Hashimoto et al., 2002a) as well as in other neuronal cells (Noh and Koh, 2000), and the most recent reports have established an essential role of NADPH oxidase in mediating signals for neuronal cell death (Noh and Koh, 2000; Hashimoto et al., 2002a, 2003).

Second, we examined the effects of cell-permeable caspase inhibitors, Ac-DEVD-CHO (DEVD), the inhibitor of caspases-3/7; Ac-YVAD-CHO (YVAD), the inhibitor of caspase-1; Ac-LEHD-CHO (LEHD), the inhibitor of caspase-9; Ac-VEID-CHO (VEID), the inhibitor of caspase-6; and Ac-IETD-CHO (IETD), the inhibitor of caspases-8/6. Figure 4A indicates the results, which revealed that DEVD and LEHD drastically suppressed Aβ-induced cell death, whereas YVAD, VEID, or IETD had no effect on Aβ-induced cell death under the same conditions. In these experiments, none of the caspase inhibitors inhibited expression of p75NTR (Fig. 4B). Therefore, it is most likely that the Aβ/p75NTR-induced neurotoxic mechanism involves caspase-9 and caspases-3/7, suggesting that this mechanism uses a typical mitochondria-mediated caspase pathway.

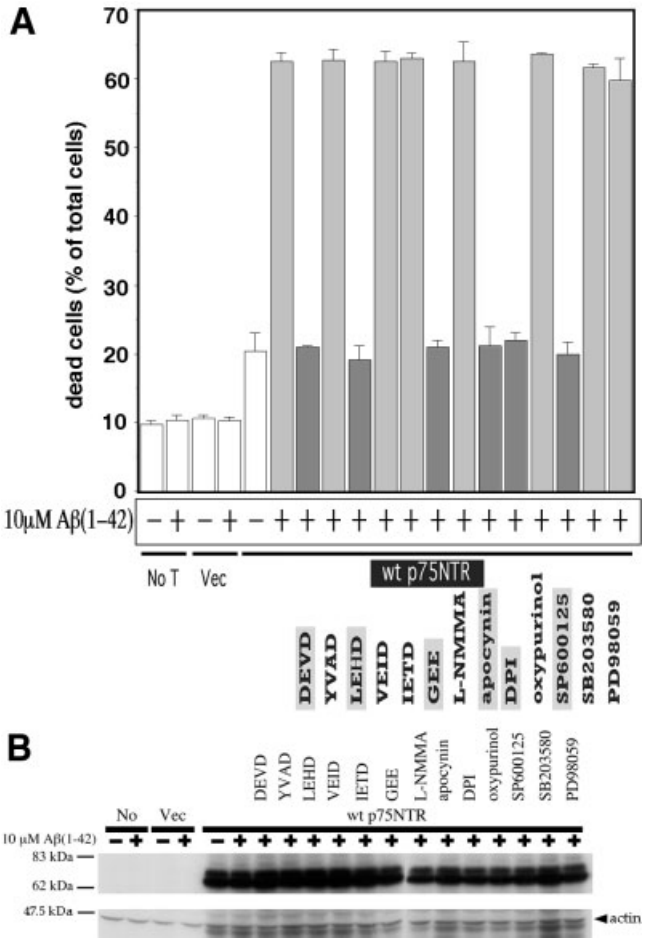

Fig. 4. Effects of antioxidants and caspase inhibitors on Aβ1–42/p75NTR-induced F11 neurohybrid cell death. **A**: F11 neurohybrid cells were transfected with p75NTR cDNA (wt p75NTR) and treated with 10 μM Aβ1–42 in the presence or absence of 100 μM caspase inhibitors (DEVD, YVAD, LEHD, VEID, and IETD), antioxidants (1 mM GEE, 1 mM L-NMMA, 300 μM apocynin, 100 μM DPI, and 100 μM oxypurinol), or MAPK family inhibitors (1 μM SP600125, 20 μM SB203580, and 50 μM PD98059). Aβ and inhibitors were added to the cell culture 24 hr after transfection. Cell mortality was measured by trypan blue exclusion assay 48 hr after transfection. **B**: Expression of p75NTR in the experiments shown in A was examined by immunoblotting using M2 anti-FLAG antibody (upper panel). In the lower panel, expression of actin in the same samples used for the upper panel was examined by anti-actin antibody (1/1,000; Sigma) as a loading control.

Third, we examined whether JNK is involved in the Aβ/p75NTR-induced neurotoxic mechanism, because recent studies (Bozyczko-Coyne et al., 2001; Morishima et al., 2001; Troy et al., 2001; Wei et al., 2002) have reported that JNK is involved in Aβ-induced neuronal cell death. F11 neurohybrid cells were transfected with p75NTR and treated with 10 μM Aβ1–42 in the presence of MAPK family inhibitors: SP600125, the specific JNK inhibitor; SB203580, the p38 MAPK inhibitor; and PD98059, the MEK/MAPK inhibitor. Figure 4A indi-

cates that SP600125 drastically inhibited Aβ-induced cell death and that SB203580 or PD98059 had no effect. Whereas SB203580 stimulated expression of transfected p75NTR somewhat, none of these MAPK family inhibitors inhibited expression of p75NTR (Fig. 4B). These data indicate that JNK is involved in the Aβ/p75NTR-induced neurotoxic mechanism.

Inhibition of Aβ/p75NTR-Induced Cell Death by HN and Other Known Rescue Factors

It has thus far been shown that (S14G)HN, termed HNG, ADNF, IGF-I, and bFGF are able to suppress Aβ-induced cell death in primary neurons at nanomolar or lower concentrations (Hashimoto et al., 2001c). We examined whether this is the case with Aβ/p75NTR-induced cell death in the neurohybrid cell system. In F11 cells transfected with p75NTR and treated with 10 μM Aβ1–42, 10 nM HNG drastically suppressed Aβ-induced cell death (Fig. 5A). In contrast, (C8A)HN, termed *HNA*, which is an inactive HN derivative (Hashimoto et al., 2001a), had no effect on Aβ/p75NTR-induced cell death, even at 10 μM (Fig. 5A). Drastic suppression was also the case with ADNF, IGF-I, and bFGF, all of which greatly inhibited Aβ/p75NTR-induced cell death (Fig. 5A). None of these factors suppressed expression of p75NTR (Fig. 5B).

Figure 5A also includes the effect of NGF, which is a natural ligand for p75NTR. When F11 neurohybrid cells were transfected with p75NTR in the absence of Aβ, NGF treatment significantly enhanced cell death. When F11 neurohybrid cells were transfected with p75NTR in the presence of NGF, 10 μM Aβ1–42 only slightly augmented the cell mortality already enhanced by NGF. Therefore, it is highly likely that Aβ and NGF trigger the same neurotoxic mechanism of p75NTR.

DISCUSSION

We have confirmed that p75NTR is able to mediate Aβ toxicity in neurohybrid cells. We characterized, based on this confirmation, the Aβ/p75NTR-induced neurotoxic mechanism, which revealed the following three points. First, the characterized mechanism was consistent with the known mechanism for Aβ neurotoxicity. The antioxidant GEE, the caspase inhibitor DEVD, and the JNK inhibitor SP600125 suppressed Aβ/p75NTR-induced neurotoxicity without reducing p75NTR expression. These results are consistent with existing reports (for review see Behl and Moosmann, 2002; see also Loo et al., 1993; Yaar et al., 1997; Allen et al., 2001; Bozyczko-Coyne et al., 2001; Morishima et al., 2001; Troy et al., 2001; Wei et al., 2002). In particular, Yaar et al. (1997) found, for the first time, that JNK is activated by binding of Aβ to p75NTR on the cell surface. The present study also showed that the $G_{i/o}$ protein inhibitor PTX blocked Aβ/p75NTR-induced neurotoxicity without reducing p75NTR expression; this is consistent with the study of Wei et al. (2002): This study also clarified that Aβ/p75NTR-induced neurotoxicity is sensitive to low concentrations of ADNF, IGF-I, bFGF, and HNG but not HNA. This is consistent with the reported characteristics of Aβ toxicity in primary neurons: Aβ-induced cell death of primary neurons is inhibited by low concentrations of ADNF, IGF-I, bFGF, and HNG but not HNA (Hashimoto et al., 2001c).

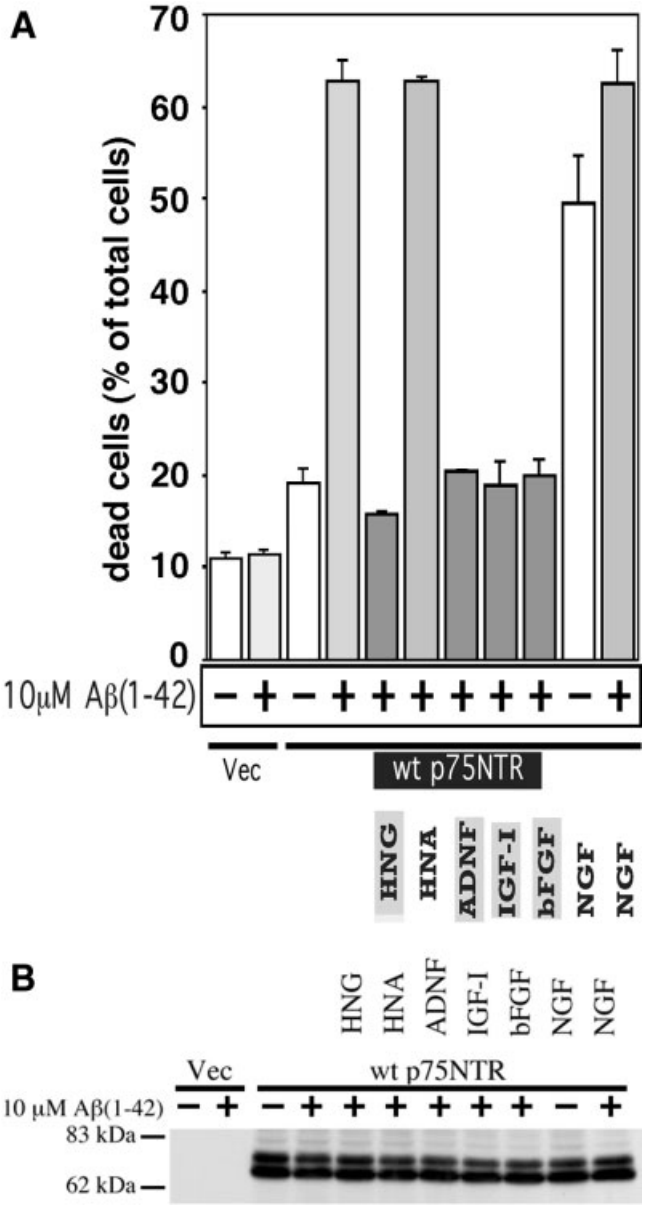

Fig. 5. Effects of HNG, IGF-1, ADNF, and bFGF on Aβ1–42/p75NTR-induced F11 neurohybrid cell death. **A**: F11 neurohybrid cells were transfected with p75NTR cDNA (wt p75NTR) or empty vector (vec) and treated with 10 μM Aβ1–42 in the presence or absence of 10 nM HNG, 10 μM HNA, 100 fM ADNF, 100 nM IGF-1, or 1 μg/ml bFGF. Treatment with 10 nM NGF was performed both with and without Aβ1–42 treatment. Aβ and inhibitors were added to the cell culture 24 hr after transfection. Cell mortality was measured by trypan blue exclusion assay 48 hr after transfection. **B**: Expression of p75NTR in the experiments shown in A was confirmed by immunoblotting using M2 anti-FLAG antibody.

Second, we have further specified the underlying mechanisms for Aβ/p75NTR-induced neurotoxicity. As for the sensitivity to GEE, this study has clarified that the potential source of ROS generation is NADPH oxidase and not NOS or XO. This result is consistent with the finding of Rabizadeh et al. (2000) that p75NTR-mediated NGF neurotoxicity is sensitive to DPI, although they did not examine p75NTR-mediated Aβ neurotoxicity. As for the details of caspase inhibitor sensitivity, Aβ/p75NTR-induced neurotoxicity was sensitive to the inhibitor of caspases-3/7 and the inhibitor of caspase-9 but resistant to the inhibitor of caspase-1, the inhibitor of caspase-6, and the inhibitor of caspases-8/6, suggesting that the Aβ/p75NTR-induced neurotoxic mechanism utilizes a typical mitochondria-mediated caspase pathway consisting of caspase-9 and caspases-3/7 in neurohybrid cells. As for PTX sensitivity, this study has specified, by using PTX-resistant G protein mutants, that G_o is the PTX target that functions as the signal mediator of Aβ/p75NTR-induced neurotoxicity.

Third, we have determined that the L401K mutation nullifies the function of p75NTR that mediates Aβ neurotoxicity. Rabizadeh et al. (2000) reported that the synthetic peptide corresponding to residues 396–409 of p75NTR can induce cellular apoptosis. Medina et al. (2002) subsequently found that substitution of Lys for Leu^{401} (L401K) abolishes the proapoptotic activity of this synthetic peptide. Nevertheless, it has not been examined whether $p75NTR^{L401K}$ is deficient in mediating neurotoxicity.

At present, it remains undetermined whether the linkage of p75NTR with G_o is direct. Considering that G protein-coupled receptors usually consist of a seven-transmembrane structure and that p75NTR is a single-transmembrane protein, it would be less likely that p75NTR directly couples G_o. However, there are several exceptional cases in which single-transmembrane receptors or receptor-like proteins are directly linked to G proteins (Murayama et al., 1990; Nishimoto et al., 1993; Gong et al., 1995; Sun et al., 1995, 1997). Among others, AβPP has been shown to interact directly with G_o, but not G_i, and furthermore to activate G_o in a phospholipid vesicle system (Okamoto and Nishimoto, 1992; Nishimoto et al., 1993; Bruillet et al., 1999). In the F11 neurohybrid cell system, as used in the present study, our earlier study showed that G_o activation by antibody-stimulated AβPP leads to activation of the JNK/NADPH oxidase/caspase-mediated cell death pathway (Hashimoto et al., 2003). Therefore, it is highly likely that, after G_o activation, Aβ/p75NTR neurotoxicity in F11 neurohybrid cells utilizes the same pathway as antibody/AβPP neurotoxicity. Therefore, p75NTR could be another exception in which a single-transmembrane protein directly couples G_o. In fact, p75NTR has a cytoplasmic region with which it can potentially interact with G proteins: the Arg^{384}-Arg^{410} region (see the introductory paragraphs). This region is included in the death domain of p75NTR, which has been shown to be responsible for Aβ neurotoxicity (Perini et al., 2002), and contains Leu^{401}, whose substitution nullified the neurotoxic function of intact p75NTR. In any event, although it is interesting, this issue is beyond the scope of the present study.

It is generally accepted that understanding the Aβ neurotoxic mechanism would contribute significantly to the development of anti-AD therapies. The present study has shown the essential role of p75NTR, G_o, and its downstream neurotoxic mechanism with regard to Aβ neurotoxicity. At present, most trials antagonizing Aβ neurotoxicity for the purpose of clinical AD treatment are to reduce Aβ production or remove Aβ burden. Based on this study, a new modality of anti-Aβ therapeutics becomes possible. For instance, screening an Aβ blocker that competes with the binding to p75NTR would provide a clinically applicable method to suppress Aβ neurotoxicity without affecting the Aβ burden in the central nervous system.

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