

Neurotoxic Mechanisms by Alzheimer's Disease-Linked N141I Mutant Presenilin 2

YUICHI HASHIMOTO, TAKAKO NIIKURA, YUKO ITO, YOSHIKO KITA, KENZO TERASHITA, and IKUO NISHIMOTO

Department of Pharmacology and Neurosciences, KEIO University School of Medicine, Shinanomachi, Shinjuku-ku, Tokyo Japan

Received August 20, 2001; accepted November 6, 2001 This article is available online at <http://jpet.aspetjournals.org>

ABSTRACT

Although it has been established that oxidative stress mediates cytotoxicity by familial Alzheimer's disease (FAD)-linked mutants of presenilin (PS)1 and that pertussis toxin inhibits cytotoxicity by FAD-linked N141I-PS2, it has not been determined whether oxidative stress is involved in cytotoxicity by N141I-PS2 or which pertussis toxin-sensitive proteins mediate the cytotoxicity. Here we report that low expression of N141I-PS2 caused neuronal cell death, whereas low expression of wild-type PS2 did not. Cytotoxicities by low and high expression of N141I-PS2 occurred through dissimilar mechanisms: the former cytotoxicity was blocked by a cell-permeable caspase inhibitor, and the latter was not. Since both mechanisms were sensitive to a cell-permeable antioxidant, we examined poten-

tial sources of reactive oxygen species in each mechanism, and found that the caspase inhibitor-sensitive neurotoxicity by N141I-PS2 was likely through NADPH oxidase and the caspase inhibitor-resistant neurotoxicity by N141I-PS2 through xanthine oxidase. Pertussis toxin greatly suppressed both toxic mechanisms by N141I-PS2, and only G_{α_o} , a neuron-enriched pertussis toxin-sensitive G protein, was involved in both mechanisms. We therefore conclude that N141I-PS2 is capable of triggering multiple neurotoxic mechanisms, which can be inhibited by the combination of clinically usable inhibitors of NADPH oxidase and xanthine oxidase. This study thus provides a novel insight into the therapeutic intervention of PS2 mutant-associated FAD.

Familial Alzheimer's disease (FAD) is caused by mutations in amyloid precursor protein (APP), presenilin (PS)1, and PS2 (Shastri and Giblin, 1999). However, how these mutant genes cause neural death, the central abnormality in Alzheimer's disease (AD), has been little understood. A clue is the finding that expression of FAD mutant APP and PS genes causes neural cell death in cultures (Guo et al., 1996, 1999; Wolozin et al., 1996; Yamatsuji et al., 1996; Zhao et al., 1997; Czech et al., 1998; Luo et al., 1999; Weihl et al., 1999; Hashimoto et al., 2000b). It has also been found that V642 mutants (V642I/F/G) of APP and K595N/M596L-APP (NL-APP) induce cytotoxicity through pertussis toxin (PTX)-sensitive G proteins (Wolozin et al., 1996; Yamatsuji et al., 1996; Giambarella et al., 1997; Hashimoto et al., 2000b). Wolozin et al. (1996) found that N141I-PS2, linked with FAD in the Volga German families, causes PC12 neuronal cell death in a PTX-

sensitive manner. It is thus important to clarify the entire array of death signals generated by FAD genes.

The mechanisms underlying mutant APP-induced neurotoxicity have been considerably defined (Yamatsuji et al., 1996; Giambarella et al., 1997; Hashimoto et al., 2000b). Also, it has been established that N141I-PS2 causes neuronal cell death (Wolozin et al., 1996; Araki et al., 2000; Hashimoto et al., 2001a, b). However, little has been known about the mechanism whereby N141I-PS2 contributes to neuronal cell death, except for mediation by PTX-sensitive G proteins (Wolozin et al., 1996). We (Hashimoto et al., 2000b) have recently established a neuronal cell system in which low levels of single-cell expression of a cDNA of interest in an ecdysone (EcD)-inducible plasmid are precisely controlled by treated EcD doses without affecting transfection efficiency. In this system, it was found that 1) high expression (≥ 3 -fold expression of endogenous APP) of wild-type (wt) APP weakly induces neuronal cell death resistant to both glutathione-ethyl-ester (GEE), a well established cell-permeable antioxidant, and Ac-DEVD-CHO (DEVD), a cell-permeable inhibitor of the apoptosis-mediating caspases; 2) low expression (< 3 -fold expression of the endogenous protein) of two FAD-linked

This work was supported in part by grants from Tomy Medico, Noevir, Japan Foundation for Neuroscience and Mental Health (Y. H.), Ono Medical Research Foundation, Keio Gijuku Academic Development Funds (Y. H. and Y. K.), and the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

ABBREVIATIONS: FAD, familial Alzheimer's disease; PS, presenilin; APP, amyloid precursor protein; NL-APP, K595N/M596L-APP; PTX, pertussis toxin; GEE, glutathione-ethyl-ester; DEVD, Ac-DEVD-CHO; APO, apocynin; DPI, diphenyleneiodonium; XO, xanthine oxidase; XO_i, XO inhibitor; ROS, reactive oxygen species; OXYP, oxypurinol; CTF, C-terminal fragment; EGFP, enhanced green fluorescent protein; wt, wild-type; FBS, fetal bovine serum; EcD, ecdysone; EtOH, ethanol; PTXr- G_{α} , PTX-resistant mutant of G protein α subunit $G_{\alpha_{11}}$, $G_{\alpha_{12}}$, $G_{\alpha_{13}}$, or G_{α_o} ; L-NAME, N^{ω} -nitro-L-arginine methyl ester.

mutant APPs (V642I-APP and NL-APP) causes high levels of GEE/DEVD-sensitive cytotoxicity; 3) low and high expressions of V642I-APP induce the same cytotoxicity; and 4) high expression of NL-APP additionally augments the GEE/DEVD-resistant cytotoxicity of wtAPP. Multiple mechanisms thus underlie neurotoxicity by even two of the FAD-linked mutants of APP. Using the same system, the present study was conducted to investigate the molecular mechanisms whereby wild-type or mutant PS2 kills neuronal cells. Herein we report that distinct sets of different toxic mechanisms underlie cytotoxicity by N141I-PS2 and wtPS2, and compare them with the toxic mechanisms of APP mutants.

Materials and Methods

wtAPP and V642I-APP cDNAs were described previously (Hashimoto et al., 2000b). wtPS2 and N141I-PS2 cDNAs were kindly provided by Dr. P. St-George Hyslop (University of Toronto, Canada) and Dr. L. D'Adamio (Albert Einstein College of Medicine, NY), respectively. These PS2 cDNAs were subcloned into pIND (Invitrogen, Carlsbad, CA), an EcD-inducible plasmid, with sequence confirmation. The pIND-encoded wtPS2 or N141I-PS2 was designated as pIND-wtPS2 or pIND-N141I-PS2, respectively. The cDNAs encoding PTX-resistant G protein α subunits (PTXr-G α : PTXr-G α_{11} , PTXr-G α_{12} , PTXr-G α_{13} , and PTXr-G α_o), described previously (Taussig et al., 1992), were kindly provided by Drs. R. Taussig (University of Michigan, MI) and T. Kozasa (University of Texas, Southwestern Medical Center, TX). EGFP cDNA (pEGFP-N1; CLONTECH, Palo Alto, CA) was also subcloned to pIND (pIND-EGFP). GEE, apocynin (4-hydroxy-3-methoxyacetophenone; APO), and diphenyleneiodonium (DPI) were from Sigma-Aldrich (St. Louis, MO), and PTX from Calbiochem-Novabiochem (San Diego, CA). Ac-DEVD-CHO was from Peptide Institute (Osaka, Japan). Ponasterone (Invitrogen) was employed as EcD. (+) and (–)BOF4272 were provided by Otsuka Pharmaceutical Factory (Naruto, Japan).

F11 cells were grown in Ham's F-12 plus 18% FBS and antibiotics. F11 cells, the hybrid cells of a rat embryonic day 13 primary cultured neuron and a mouse neuroblastoma, are one of the best models for primary cultured neurons, as described previously (Yamatsuji et al., 1996). F11 cells over-expressing both EcR and RXR (F11/EcR cells) were established using the coexpression vector pVgRXR (Invitrogen) and Zeocin selection. For transient transfection of the pIND plasmids, F11/EcR cells were seeded at 7×10^4 cells/well in a 6-well plate and cultured in Ham's F-12 plus 18% FBS for 12 to 16 h, and were transfected with EcD-inducible pIND plasmids [1 μ g pIND plasmids, 2 μ l LipofectAMINE, and 4 μ l PLUS reagent (Invitrogen)] in the absence of serum for 3 h. After subsequent incubation with Ham's F-12 plus 18% FBS for 12 to 16 h, cells were cultured with or without inhibitors in Ham's F-12 plus 10% FBS for 2 h, and EcD was then added to the media (without medium change). Cell mortality was measured by Trypan blue exclusion assay at 72 h after the onset of EcD treatment. Fluorescence of the cells transfected with pIND-EGFP was assessed by transfecting this plasmid (1 μ g of plasmid, 2 μ l of LipofectAMINE, and 4 μ l of PLUS reagent) in the absence of serum for 3 h. After subsequent incubation with Ham's F-12 plus 10% FBS for 69 h, fluorescence intensity was assessed by measuring the fluorescence intensity of randomly chosen cells in each transfection with standardization by the cell area and calculating the mean \pm S.D. of these values for each transfection. Immunoblot analysis of expressed PS2 constructs was performed using anti-PS2 antibody 2192 (1:500 dilution; Cell Signaling Technology, Beverly, MA), which can detect low endogenous levels of PS2 holoprotein at 54 kDa, and the expression was quantified, essentially according to the method described previously (Hashimoto et al., 2000b). Mouse primary neuronal cultures, in which >98% of cells were neurons, were prepared as described previously (Sudo et al., 2000).

Trypan blue exclusion assay was described in detail previously

(Hashimoto et al., 2000b). The basal death rates with or without empty pIND vector transfection and with or without EcD treatment indicated the actual fraction of dead cells, but not artificial cell death occurring after detaching cells, as in situ staining of Trypan blue-positive cells indicated the presence of similar fractions of basally occurring cell death (Hashimoto et al., 2000b; Sudo et al., 2000). It has been demonstrated that the cell mortality assessed by this assay is quantitatively confirmed by cell viability assay (Wako Pure Chemicals, Osaka, Japan), using WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt] (Hashimoto et al., 2000a).

All of the experiments described in this study were repeated at least three times with independent transfections and treatments, each of which yielded essentially the same result. Statistical analysis was performed with Student's *t* test, in which *p* < 0.05 was assessed as significant.

Results

Dose-Dependent Expression of the pIND Construct by EcD. F11/EcR cells were transfected with pIND-EGFP cDNA with subsequent treatment with various concentrations of EcD. The transfection efficiency was very constant at 65 to 70%, as reported previously (Hashimoto et al., 2000b). The results, shown in the left panel of Fig. 1A, indicate that the cellular expression of the construct was augmented in a linear relationship with the treated doses of EcD. Thus, as expected, the pIND construct was expressed in an EcD-dose-dependent and -proportional manner. It was also indicated that neither treatment with 100 μ M DEVD, 1 mM GEE, nor 1 μ g/ml PTX affected EcD-dependent expression of pIND-EGFP (Fig. 1A, middle panel).

When wtPS2 cDNA in pIND was transfected, immunoblot analysis of PS2 holoprotein confirmed that there was a strict linear relationship between the EcD dose and expressed PS2 holoprotein, as shown in the right panel of Fig. 1A (black triangles). Although endogenous PS2 holoprotein in F11/EcR cells was undetectable, as described previously (Hashimoto et al., 2001b), 20 and 40 μ M EcD caused \sim 2 and \sim 3.5-fold expression of wtPS2 relative to that of wtPS2 induced by 10 μ M EcD. This is consistent with our earlier study (Hashimoto et al., 2000b) that when wtAPP cDNA in pIND was transfected and expressed by EcD in the same F11/EcR cells, there was a strict linear relationship between the EcD dose and expressed wtAPP.

Expression of N141I-PS2 holoprotein was not linearly augmented by EcD, and reached saturation by \geq 10 μ M EcD (blue circles in the right panel of Fig. 1A). Considering the present result that N141I-PS2 induction by \geq 10 μ M EcD caused DEVD-sensitive cell death (see below) and from the literature that PS2 holoprotein is cleaved by DEVD-sensitive caspases (Vito et al., 1997), we reasoned that in this system, N141I-PS2 was induced by EcD similarly to the induction of wtPS2, but degraded through mechanisms involving caspase activation by expressed N141I-PS2 itself, resulting in certain balanced expression. In accordance with this idea, in the presence of 100 μ M DEVD, an established cell-permeable inhibitor of caspases, expression of N141I-PS2 became linear in relationship to EcD concentrations on the line similar to that of wtPS2 expression (red squares in the right panel of Fig. 1A). Under the same conditions, expression of wtPS2 was not affected by DEVD (data not shown). These data suggest that N141I-PS2 in pIND, like wtPS2 in the same

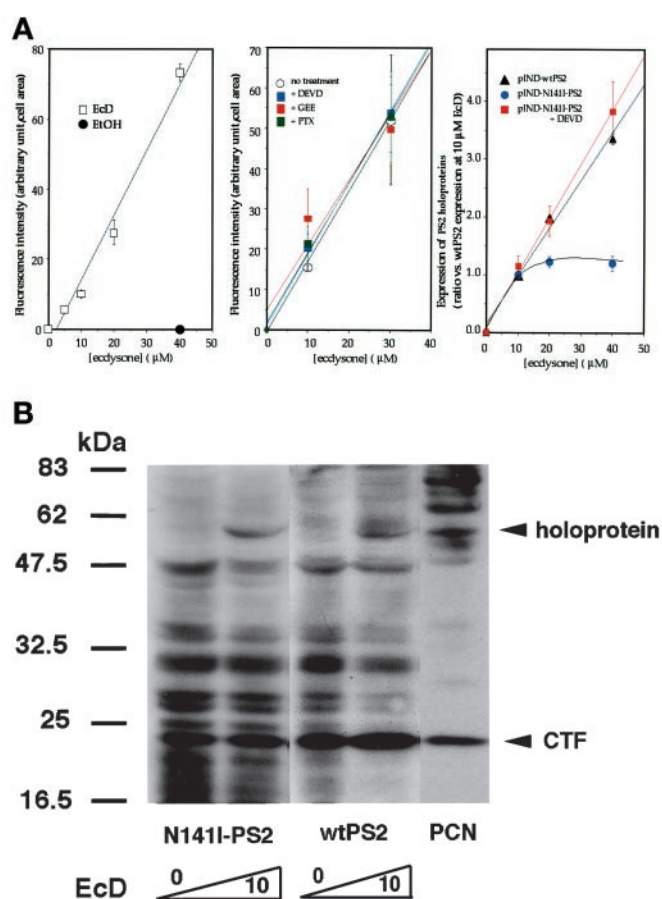


Fig. 1. EcD dose-dependence of encoded protein expression in F11/EcR cells transfected with pIND constructs. **A**, in the left panel, F11/EcR cells were transfected with pIND-EGFP and treated with increasing concentrations of EcD. Fluorescence intensity of transfected cells was measured as described under *Materials and Methods*. In the middle panel, F11/EcR cells were transfected with pIND-EGFP and treated with increasing doses of EcD in the presence or absence of 100 μM DEVD, 1 mM GEE, or 1 μg/ml PTX. In the right panel, F11/EcR cells were transfected with pIND-wtPS2 or pIND-N141I-PS2 and treated with increasing concentrations of EcD in the presence or absence of 100 μM DEVD. Expressed PS2 holoproteins were examined by immunoblot analysis 72 h after transfection. The ratios of the 54-kDa PS2 immunoreactivity for the actin immunoreactivity in the same samples were measured, as described previously (Hashimoto et al., 2000b), and they are indicated as a function of the concentration of EcD, in which the PS2 expression by 10 μM EcD is set as 1.0. All values indicate means \pm S.D. of at least three independent experiments. **B**, immunoblotting of PS2 constructs induced in transfected F11/EcR cells and endogenous PS2 in mouse primary cultured neurons. F11/EcR cells were transfected with pIND-wtPS2 or pIND-N141I-PS2 and treated with or without 10 μM EcD. Expressed PS2 proteins were examined by immunoblot analysis (20 μg/lane) 72 h after transfection. The extreme right lane indicates PS2 immunoblotting of a cell lysate (20 μg/lane) from primary cultured neurons (PCN). The top and bottom arrowheads indicate the holoprotein and the CTF of PS2 proteins, respectively.

vector, was induced in proportion to the EcD concentration, and that N141I-PS2, but not wtPS2, was degraded by activating DEVD-sensitive mechanisms. This is again consistent with our earlier study that 1) in the same system, expression of V642I-APP or NL-APP in pIND, both of which can activate DEVD-sensitive caspases, is not linearly augmented and reaches saturation by ≥ 10 μM EcD; 2) their expression is linearly augmented by EcD in the presence of DEVD; and 3) wtAPP is expressed proportionally to the EcD concentration (Hashimoto et al., 2000b). Combined with the quantitative

validity of the employed EcD/EcR-inducible expression system (No et al., 1996) and the observed linearity of the EcD-induced EGFP expression in pIND-EGFP-transfected cells (Fig. 1A, left panel), these data support that the PS2 constructs (wtPS2 and N141I-PS2) were induced by EcD dose dependently.

In this study, we thus defined low expression as that elicited by 10 μM EcD and high expression as that elicited by 40 μM EcD, as in our earlier study for inducible APP constructs (Hashimoto et al., 2000b). In this system, the low expression level of PS2 holoproteins (wtPS2 and N141I-PS2) was comparable to the endogenous expression level of the PS2 holoprotein in mouse primary cultured neurons (Fig. 1B). Although anti-PS2 antibody 2192 was against the C terminus of PS2 and can recognize both the holoprotein and the C-terminal fragment (CTF) of PS2, we were not able to clearly specify the CTF (deduced size, 23 kDa) of expressed PS2 proteins because the 2192 antibody was the only one available that could detect endogenous levels of PS2 (no other antibodies tested could detect PS2 holoprotein expression by 40 μM EcD in the present system), and F11/EcR cells express quite a few transfection-independent proteins recognized by this antibody at 20 to 25 kDa. However, this antibody clearly detected the 54-kDa holoprotein of PS2. In addition, comparison with the immunoblot result from primary neurons suggests that the strong 23-kDa immunoreactive band was apparently the CTF of PS2. It was thus likely that in this system, the quantity of the CTF of PS2 was not critically affected by transfected induction of PS2 constructs (wtPS2 and N141I-PS2); only the quantity of the holoproteins was controlled by the induction. Also, the observed linear relationship between EcD and expressed PS2 holoproteins (wtPS2 in the presence or absence of DEVD and N141I-PS2 in the presence of DEVD), as linear as that between EcD and EGFP in the same vector, suggests that the cleavage of full-length PS2 was not affected by EcD.

Effect of Low and High Expression of N141I-PS2 on Neuronal Cell Death. When N141I-PS2 cDNA was induced by EcD in transfected F11/EcR cells, cell death robustly occurred in a manner dependent on the concentration of EcD (Fig. 2). In pIND-N141I-PS2-transfected cells, 10 μM EcD caused death in nearly 60% of cells in a saturated manner after 72 h. In contrast, the vehicle ethanol (EtOH) caused no increase in cell mortality in either case. Treatment of non-transfected F11/EcR cells or vector-transfected F11/EcR cells with or without EcD resulted in low cell mortality ($\sim 10\%$) for 72 h, which was estimated to be the basal death rate of these cells. The top panel of Fig. 2 indicates the stability of low death rates under the negative conditions. In all dose-response experiments shown in each figure in this study, we performed the experiments measuring cell mortality in the presence or absence of 40 μM EcD without transfection and in the presence or absence of 40 μM EcD with empty pIND transfection, both of which were constantly as low as the basal cell mortality in the absence of EcD with pIND-PS construct transfection (Fig. 2, top panel). The low basal death rates after lipofection and small variations in the stimulated death rates were mainly attributed to the short lipofection period and subsequent serum treatment in the present protocol.

As transfection efficiency was constantly 65 to 70%, as described previously (Hashimoto et al., 2000b), it followed

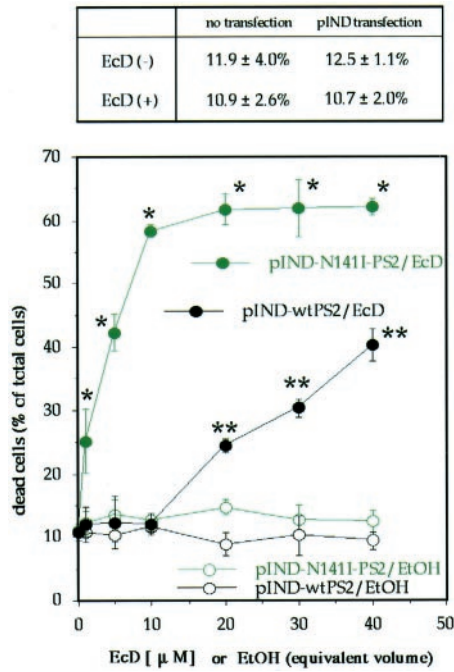


Fig. 2. Death induced by EcD in F11/EcR cells transfected with pIND-N141I-PS2. Dose-dependent increase by EcD in mortality of cells transfected with pIND-N141I-PS2. F11/EcR cells were transfected with pIND-N141I-PS2 (green circles) or pIND-wtPS2 (black circles) and treated with increasing concentrations of EcD (filled circles) or equivalent volumes of EtOH (open circles). Cell mortality was determined by Trypan blue exclusion assay 72 h after the onset of EcD treatment. In the upper panel, cell mortalities in negative controls simultaneously performed with the experiments shown in the lower panel are indicated. F11/EcR cells were transfected with (pIND transfection) or without (no transfection) empty pIND plasmid and then treated with (+) or without (-) 40 μM EcD for 72 h, and cell mortality was measured. *, significant versus basal cell death rates in the upper panel and cell death rates by pIND-N141I-PS2 transfection in the absence of EcD. **, significant versus basal cell death rates and cell death rates by pIND-wtPS2 transfection in the absence of EcD. All cell death rates by pIND-wtPS2 transfection in the presence of EcD, shown in the lower panel, are significantly lower than those by pIND-N141I-PS2 transfection in the presence of EcD. All values indicate means ± S.D. of at least three independent experiments.

that expression of N141I-PS2 by ≥10 μM EcD induced death in most of the transfected cells after 72 h. Expression of wtPS2 resulted in different dose-response curves for death (Fig. 2, black filled circles). EcD caused little death in pIND-wtPS2-transfected cells at ≤10 μM, and augmented the death rates dose dependently only at ≥20 μM. Although EtOH caused no increase in cell mortality in either case (Fig. 2, black open circles), expression of wtPS2 by ≥30 μM EcD exerted significant cytotoxicity, but to lesser degrees than the cytotoxicity by the same concentrations of EcD in cells transfected with pIND-N141I-PS2. Thus, low expression of N141I-PS2 robustly caused cell death, whereas wtPS2 was toxic at only high expression.

Effect of Ac-DEVD-CHO on Cytotoxicity by N141I-PS2. We next investigated whether DEVD affects cytotoxicity by N141I-PS2. The dose-response relationship for EcD-stimulated cytotoxicity in pIND-N141I-PS2-transfected cells was examined in the presence or absence of 100 μM DEVD. Mortality of cells transfected with or without pIND treated with or without EcD in the presence of 100 μM DEVD was ~10% (Fig. 3, left top panel). The curve of N141I-PS2-induced cytotoxicity in the presence of DEVD was intermediate between the cytotoxicity curve of N141I-PS2 and that of

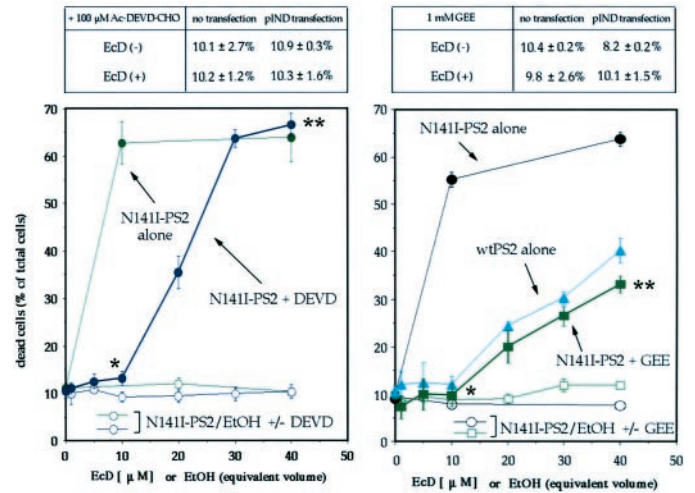


Fig. 3. Effect of Ac-DEVD-CHO or GEE on cell death by pIND-N141I-PS2. In the left panel, F11/EcR cells were transfected with pIND-N141I-PS2 and treated with increasing concentrations of EcD (filled circles) or equivalent volumes of EtOH (open circles) in the presence (blue circles) or absence (green circles) of 100 μM DEVD. Cell mortality was determined 72 h after the onset of EcD treatment. In the right panel, F11/EcR cells were transfected with pIND-N141I-PS2 and treated with increasing concentrations of EcD (filled symbols) or equivalent volumes of EtOH (open symbols) in the presence (green squares) or absence (black circles) of 1 mM GEE. Cell mortality was similarly determined. The dose-response curve of death by wtPS2, shown in Fig. 2, is overlaid to allow a direct comparison (cyan filled triangles). In the top panels, cell mortalities in the presence of 100 μM DEVD (left) or 1 mM GEE (right) in negative controls simultaneously performed with the experiments shown in the corresponding bottom panels are indicated. * and **, significant and not significant, respectively, versus cell death rates by pIND-N141I-PS2 transfection in the presence of corresponding concentrations of EcD in the absence of DEVD (left panel) or GEE (right panel). All values indicate means ± S.D. of at least three independent experiments.

wtPS2. Cytotoxicity by low expression of N141I-PS2 was blocked by DEVD, whereas cytotoxicity by high expression of N141I-PS2 was DEVD-resistant (Fig. 3, left panel). These data indicate that cytotoxicity by N141I-PS2 consists of two components in addition to wtPS2 cytotoxicity: DEVD-sensitive cytotoxicity by low expression of N141I-PS2 and DEVD-resistant cytotoxicity by its high expression.

Effect of GEE on Cytotoxicity by N141I-PS2. The sensitivity of N141I-PS2-induced cytotoxicity was different for GEE. The dose-response curve of N141I-PS2 cytotoxicity was altered sharply downward by 1 mM GEE and became virtually identical to the dose-response curve of wtPS2 cytotoxicity (Fig. 3, right panel). This result suggests that N141I mutation-specific cytotoxicity was overlaid on wtPS2 cytotoxicity and was sensitive to GEE. Combining these data with the aforementioned DEVD data further suggests that in addition to the GEE/DEVD-resistant mechanism of wtPS2 cytotoxicity, N141I-PS2 triggered two distinct cytotoxic mechanisms, both different from the mechanism for wtPS2-induced cytotoxicity. One, activated by low expression of N141I-PS2, was GEE/DEVD-sensitive. Another, activated by its high expression, was GEE-sensitive/DEVD-resistant.

Effect of PTX on Cell Death by N141I-PS2. We next investigated whether PTX, an inhibitor of heterotrimeric G proteins, affects cytotoxicity by N141I-PS2, as reported by Wolozin et al. (1996), and if so, which mechanism for cell death, by low or high expression, is inhibited by PTX. When F11/EcR cells transfected with pIND-N141I-PS2 were treated with EcD in the presence of 1 μg/ml PTX, EcD-

stimulated cytotoxicity was drastically suppressed, even below the dose-response curve of wtPS2 cytotoxicity (Fig. 4, left panel). These data suggest that cytotoxicity by N141I-PS2 (both low and high expression) is PTX-sensitive, and that cytotoxicity by wtPS2 would also be sensitive to PTX.

We thus examined the PTX sensitivity of wtPS2 cytotoxicity. As expected, cytotoxicity by wtPS2 was inhibited by PTX, to a level equivalent to the dose-response curve of N141I-PS2 cytotoxicity in the presence of PTX (Fig. 4, right panel). Thus, both N141I-PS2-induced and wtPS2-induced cytotoxicities were sensitive to PTX.

Effect of Specific Xanthine Oxidase Inhibitors on Cell Death by N141I-PS2. Not only to confirm the GEE-sensitive cytotoxicity by N141I-PS2, but also to specify the underlying mechanism, we examined the effect of oxypurinol (OXYP), a cell-permeable xanthine oxidase inhibitor (XOI). Xanthine oxidase (XO) has been established to be a source of the reactive oxygen species (ROS) involved in various signaling pathways. The left panel of Fig. 5 indicates that the dose-response curve of N141I-PS2 cytotoxicity in the presence of 100 μ M OXYP was virtually identical to its dose-response curve in the absence of OXYP. This result demonstrates that GEE/DEVD-sensitive cytotoxicity by N141I-PS2 was resistant to OXYP, suggesting that the GEE target source of ROS in this mechanism was different from XO. Likewise, 100 μ M OXYP had no effect on GEE/DEVD-sensitive cytotoxicity by V642I-APP under the same conditions (data not shown), consistent with the notion that N141I-PS2 and V642I-APP share the same mechanism for the GEE/DEVD-sensitive cytotoxicity.

As noted above, high expression of N141I-PS2 triggered another mechanism causing GEE-sensitive/DEVD-resistant cell death. We next examined whether this component of N141I-PS2 cytotoxicity was sensitive to OXYP. This component became visible, when cells were treated with 100 μ M DEVD (see above and Fig. 5, left panel, cyan filled circles). We hence examined whether OXYP affects N141I-PS2-in-

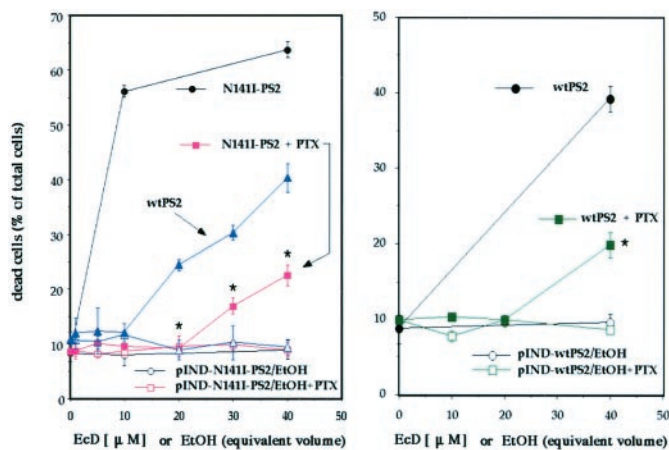


Fig. 4. Effect of PTX on cell death by pIND-N141I-PS2. F11/EcR cells were transfected with pIND-N141I-PS2 (left panel) or pIND-wtPS2 (right panel), and treated with increasing concentrations of EcD (filled symbols) or equivalent volumes of EtOH (open symbols) in the presence (squares) or absence (black symbols) of 1 μ g/ml PTX. Cell mortality was determined at 72 h after the onset of EcD treatment. The dose-response curve of death by wtPS2 is overlaid in the left panel to allow a direct comparison (blue filled triangles). * in both panels, significant versus cell death rates by pIND-N141I-PS2 transfection or pIND-wtPS2 transfection in the presence of cognate concentrations of EcD in the absence of PTX. All values indicate means \pm S.D. of at least three independent experiments.

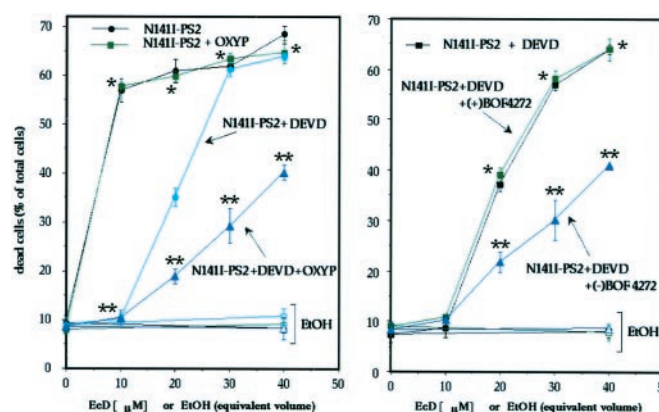


Fig. 5. Effect of XOIs on cell death by pIND-N141I-PS2. In the left panel, F11/EcR cells were transfected with pIND-N141I-PS2 and treated with increasing concentrations of EcD (filled symbols) or equivalent volumes of EtOH (open symbols) in the presence or absence (black symbols) of 100 μ M DEVD (cyan circles), 100 μ M OXYP (green squares), or both 100 μ M DEVD and 100 μ M OXYP (blue triangles). Cell mortality was similarly determined at 72 h after the onset of EcD treatment. In the right panel, F11/EcR cells were transfected with pIND-N141I-PS2 and treated with increasing concentrations of EcD (black filled squares) or equivalent volumes of EtOH (black open squares) in the presence of 100 μ M DEVD. Cell mortality was similarly determined at 72 h after the onset of EcD treatment. The green filled squares indicate the death rates of pIND-N141I-PS2-transfected cells treated with EcD, 100 μ M DEVD, and 100 nM (+)BOF4272. The blue filled triangles indicate the death rates of pIND-N141I-PS2-transfected cells treated with EcD, 100 μ M Ac-DEVD-CHO, and 100 nM (-)BOF4272. ** and * in the left panel, significant and not significant versus cell death rates by pIND-N141I-PS2 transfection in the presence of corresponding concentrations of EcD in the presence of DEVD or in all absence, respectively. ** and * in the right panel, significant and not significant, respectively, versus cell death rates by pIND-N141I-PS2 transfection in the presence of corresponding concentrations of ≥ 20 μ M EcD in the presence of DEVD. All values indicate means \pm S.D. of at least three independent experiments.

duced cell death in the presence of DEVD. In the presence of 100 μ M DEVD, 100 μ M OXYP repressed the cytotoxicity curve of N141I-PS2 down to a level equivalent to the cytotoxicity curve of wtPS2 (Fig. 5, left panel), indicating that the GEE-sensitive/DEVD-resistant cytotoxicity by N141I-PS2 was abolished by OXYP. Although OXYP has a direct superoxide-scavenging action (Das et al., 1987), it was unlikely that OXYP suppressed this GEE-sensitive mechanism by high expression of N141I-PS2 through such a scavenging effect, because the same concentration of OXYP did not affect other GEE-sensitive mechanisms activated by V642I-APP (data not shown) or low expression of N141I-PS2 (Fig. 5, left panel).

To confirm that the source of ROS involved in the GEE-sensitive/DEVD-resistant cytotoxicity by N141I-PS2 is XO, we examined the effect of (-)BOF4272, a specific inhibitor of XO, which has no direct scavenging effect on superoxide anions (Suzuki et al., 1998). We also used (+)BOF4272, an inactive enantiomer as a negative control that does not block XO (Suzuki et al., 1998). In the presence of 100 μ M DEVD, the dose-response curve of N141I-PS2 cytotoxicity was suppressed by 100 nM (-)BOF4272 down to the dose-response curve of wtPS2 cytotoxicity (Fig. 5, right panel). The same concentration of (+)BOF4272 had no effect under the same conditions. These data demonstrate that GEE-sensitive/DEVD-resistant death by high expression of N141I-PS2 is sensitive to XOIs.

Effect of Specific NADPH Oxidase Inhibitors on Cell Death by N141I-PS2. To further specify the underlying

mechanism for GEE-sensitive death by N141I-PS2, we examined the effect of APO (acetovanillone or apocynin), a cell-permeable NADPH oxidase inhibitor (Dodd-O and Pearce, 2000). NADPH oxidase is the major enzyme that generates superoxide in a number of systems, including a stress-transducing system in neurons (Noh and Koh, 2000; <http://www.j-neurosci.org/cgi/content/full/20/23/RC111>), and APO is a specific inhibitor of this enzyme. The basal death rates of F11/EcR cells transfected with or without pIND vector in the presence or absence of EcD were not affected by either 100 μ M DEVD or 300 μ M APO (Fig. 6, upper panels), or by a combination of the two (data not shown). In the presence of 300 μ M APO, the dose-response curve of N141I-PS2 cytotoxicity was greatly suppressed and became virtually identical to its dose-response curve in the presence of DEVD (Fig. 6, left lower panel). This result indicates that GEE/DEVD-sen-

sitive cytotoxicity by N141I-PS2 is APO-sensitive. It is thus highly likely that the GEE target source of ROS in this mechanism is NADPH oxidase, because NADPH oxidase is the only enzyme that is inhibited by 300 μ M APO, which cannot affect any of the other superoxide-generating enzymes (t'Hart and Simons, 1992). As was the case with N141I-PS2 cytotoxicity, GEE/DEVD-sensitive cytotoxicity by V642I-APP was suppressed by APO to the level of wtAPP cytotoxicity (Fig. 6, right bottom panel). In contrast, L-NAME, an inhibitor of NO synthase, had no effect on cytotoxicity by N141I-PS2 or V642I-APP in the same system (data not shown), confirming the action specificity of APO. These data lend additional credence to the notion that N141I-PS2 and V642I-APP share the same mechanism for the GEE/DEVD-sensitive cytotoxicity.

We next examined whether DPI reproduces the APO inhibition of N141I-PS2-induced cytotoxicity. DPI is another specific NADPH oxidase inhibitor with a structure and an action mechanism both different from those of APO, and exerts saturated inhibition at above 20 μ M (O'Donnell et al., 1993). As shown in Fig. 6 (left bottom panel), the cytotoxicity curve of N141I-PS2 was suppressed by 100 μ M DPI down to a level equivalent to the cytotoxicity curves of N141I-PS2 in the presence of APO or DEVD. This finding suggests that treatment with DPI precisely reproduced the action of APO. This was also the case with DPI suppression of V642I-APP-induced cell death (Fig. 6, right bottom panel). DPI suppressed the cytotoxicity curve of V642I-APP to a level similar to the curve suppressed by APO. These data confirm that NADPH oxidase is involved in GEE/DEVD-sensitive death by low expression of N141I-PS2 and by low and high expression of V642I-APP.

It should be emphasized that the suppression of N141I-PS2 cytotoxicity by APO or DPI was intermediate between the cytotoxicity curve of N141I-PS2 and that of wtPS2. In contrast, APO or DPI suppressed V642I-APP cytotoxicity to the level of wtAPP cytotoxicity. This observation is again consistent with the notion that in addition to the wild-type construct cytotoxicity, cytotoxicity by N141I-PS2 has two components and cytotoxicity by V642I-APP has but a single component.

Effect of PTX-Resistant Mutants of $G\alpha$ on Cell Death by N141I-PS2 in the Presence of PTX. As explained above, cytotoxicity by N141I-PS2 (both low and high expression) was inhibited by PTX. We thus investigated which PTX-sensitive G proteins are involved. The PTX-sensitive G proteins potentially involved include $G\alpha_o$, $G\alpha_{i1}$, $G\alpha_{i2}$, and $G\alpha_{i3}$. It was expected that $G\alpha_o$ mediates cytotoxicity by low expression of N141I-PS2, because cytotoxicity by V642I-APP is mediated by $G\alpha_o$ (Yamatsuji et al., 1996; Giambarella et al., 1997), and cytotoxicity by low expression of N141I-PS2 shares a number of pharmacological characteristics (GEE-sensitive, DEVD-sensitive, APO/DPI-sensitive, XO-resistant, and L-NAME-resistant) with cytotoxicity by V642I-APP. In contrast, it remained totally unknown which PTX-sensitive proteins mediate cell death by high expression of N141I-PS2.

To clarify these issues, we used PTX-resistant mutants of G protein α subunits (PTXr- $G\alpha$). They have a mutation at the fourth Cys from the extreme C terminus and can mediate the originally coupled receptor signals, even in the presence of PTX (Taussig et al., 1992). We transfected pIND-N141I-PS2

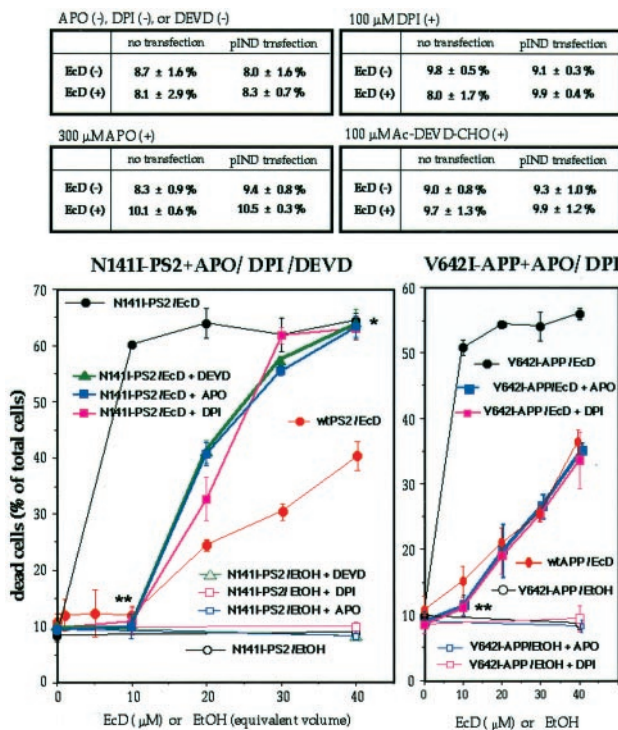


Fig. 6. Effect of NADPH oxidase inhibitors on cell death by pIND-N141I-PS2. In the lower panels, F11/EcR cells were transfected with pIND-N141I-PS2 (left) or pIND-V642I-APP (right) and treated with increasing concentrations of EcD (filled symbols) or equivalent volumes of EtOH (open symbols) in the presence or absence of 300 μ M APO, 100 μ M DPI, or 100 μ M DEVD. Cell mortality was similarly determined at 72 h after the onset of EcD treatment. In the upper panels, cell mortalities in negative controls are indicated. F11/EcR cells were transfected with (pIND transfection) or without (no transfection) empty pIND plasmid and then treated with (+) or without (−) 40 μ M EcD in the presence or absence of 300 μ M APO, 100 μ M DPI, or 100 μ M DEVD for 72 h, and cell mortality was similarly measured. In the left lower panel, the dose-response curve of wtPS2-induced death, measured in Fig. 2, is superimposed for ease of comparison (red circles). In the right lower panel, the dose-response curve of EcD-dependent death of cells transfected with pIND-wtAPP is also indicated (red circles). * in the left panel, not significant versus cell death rates by pIND-N141I-PS2 transfection in the presence of 10 μ M EcD alone. ** in the left panel, not significant versus cell death rates by pIND-N141I-PS2 transfection in the presence of 10 μ M EcD alone. All cell death rates by pIND-V642I-APP transfection (left panel) or by pIND-V642I-APP transfection (right panel) in the presence of EcD in the presence of APO or DPI are significantly lower than those by pIND-V642I-APP transfection in the presence of corresponding concentrations of EcD alone and differ not significantly from each other. All values indicate means \pm S.D. of at least three independent experiments.

or pIND-V642I-APP with or without each of the four PTXr- $G\alpha$ cDNA into F11/EcR cells, treated the transfected cells with low or high doses of EcD in the presence of PTX, and examined whether cell death occurred (Fig. 7). In the

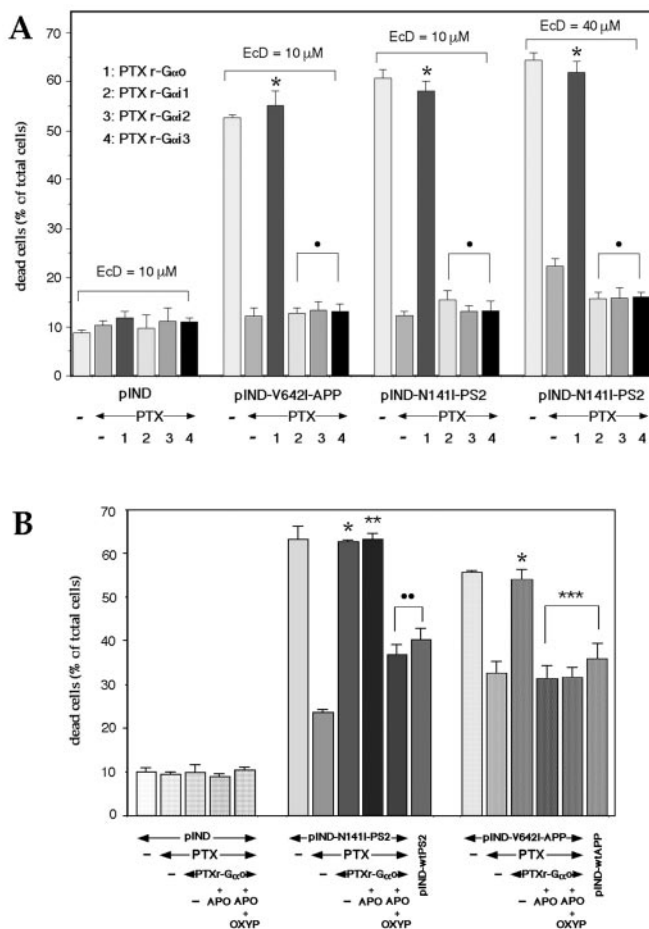


Fig. 7. Effect of PTXr- $G\alpha$ subunits on cytotoxicity by N141I-PS2 and V642I-APP. A, effect of each PTXr- $G\alpha$ on cytotoxicity by low and high expression of N141I-PS2 and by low expression of V642I-APP. F11/EcR cells were transfected with either pIND, pIND-N141I-PS2, or pIND-V642I-APP with each PTXr- $G\alpha$ cDNA (1: PTXr- $G\alpha_0$; 2: PTXr- $G\alpha_1$; 3: PTXr- $G\alpha_2$; 4: PTXr- $G\alpha_3$) or an empty plasmid (–) and were cultured with or without 1 μ g/ml PTX in the presence of 10 or 40 μ M EcD. The basally occurring cell death was at ~10% in the absence of EcD, as was the case in all other experiments shown in this study. * and •, significant and not significant increases, respectively, versus cell death rates by pIND-FAD gene transfection in the presence of indicated concentrations of EcD in the presence of PTX without transfection of PTXr- $G\alpha$ cDNA. All values indicate means \pm S.D. of at least three independent experiments. B, effect of APO and OXYP on PTXr- $G\alpha$ -restored cytotoxicity of highly induced N141I-PS2 or V642I-APP in the presence of PTX. F11/EcR cells were transfected with either pIND, pIND-N141I-PS2, or pIND-V642I-APP with PTXr- $G\alpha_0$ or an empty plasmid, and were treated with 40 μ M EcD with or without 1 μ g/ml PTX in the presence or absence of APO or OXYP. As controls, cytotoxicities by 40 μ M EcD-induced wtPS2 or wtAPP were indicated as pIND-wtPS2 or pIND-wtAPP at the extreme right columns in the middle and right groups. The basally occurring cell death was at ~10% in the absence of EcD under similar conditions. *, significant versus pIND-FAD gene transfection in the presence of 40 μ M EcD in the presence of PTX without transfection of PTXr- $G\alpha$. **, not significant versus pIND-N141I-PS2 transfection in the presence of 40 μ M EcD in the presence of PTX alone with transfection of PTXr- $G\alpha_0$. •, significant versus pIND-N141I-PS2 transfection in the presence of 40 μ M EcD in the presence of PTX and APO with transfection of PTXr- $G\alpha_0$. ••, significant versus pIND-V642I-APP transfection in the presence of 40 μ M EcD in the presence of PTX alone with transfection of PTXr- $G\alpha_0$. •••, significant versus pIND-V642I-APP transfection in the presence of 40 μ M EcD in the presence of PTX alone with transfection of PTXr- $G\alpha_0$. All values indicate means \pm S.D. of at least three independent experiments.

presence or absence of PTX, single transfection with each PTX-resistant mutant cDNA did not significantly increase cell mortality in the absence of cotransfected pIND-N141I-PS2 or pIND-V642I-APP (Fig. 7A), as described previously (Hashimoto et al., 2000a). However, in cells transfected with pIND-N141I-PS2 or pIND-V642I-APP, 10 μ M EcD killed cells cotransfected with PTX-resistant mutant $G\alpha_0$ (Fig. 7A). Under the same conditions, however, 10 μ M EcD did not augment mortality of cells cotransfected with any of the other PTX-resistant mutants of the G_i family members. These data demonstrate that low expression of N141I-PS2 or V642I-APP causes neuronal cell death only through $G\alpha_0$, but not other G_i family G proteins.

More interesting results were that in the presence of pIND-N141I-PS2 transfection, 40 μ M EcD augmented mortality of cells cotransfected only with PTXr- $G\alpha_0$, but not with any other PTX-resistant G_i mutants. Each PTXr- $G\alpha$ protein was expressed similarly, and its expression was not affected by PTX (data not shown), as described previously (Hashimoto et al., 2000a). These findings provide definite evidence that 1) $G\alpha_0$ mediates cytotoxicities by both low and high expression of N141I-PS2; 2) none of $G\alpha_i$ can mediate cell death by either low or high expression of N141I-PS2; and 3) cytotoxicity by V642I-APP is mediated by $G\alpha_0$, but not other G_i family members, as suggested by earlier studies (Nishimoto et al., 1993; Yamatsuji et al., 1996; Brouillet et al., 1999).

Therefore, two possibilities arose. One was that the observed $G\alpha_0$ -mediated cell death by high expression of N141I-PS2 is only through APO-sensitive cytotoxicity due to $G\alpha_0$ -mediated cell death by low expression of N141I-PS2. The other was that the $G\alpha_0$ -mediated cell death by high expression of N141I-PS2 is a combination of two cytotoxic mechanisms: APO- and OXYP-sensitive cytotoxicities. We therefore analyzed whether the PTXr- $G\alpha_0$ -restored cytotoxicity by high expression of N141I-PS2 consists only of APO-sensitive cytotoxicity or consists of both APO- and OXYP-sensitive cytotoxicities, in addition to wtPS2 cytotoxicity. As shown in Fig. 7B, to inhibit the PTXr- $G\alpha_0$ -restored cytotoxicity by high expression of N141I-PS2 to the level of wtPS2 cytotoxicity, both APO and OXYP were required. This result demonstrates that high expression of N141I-PS2 causes both APO- and OXYP-sensitive cytotoxicities through G_0 . In contrast, the PTXr- $G\alpha_0$ -restored cytotoxicity by high expression of V642I-APP was simply sensitive to APO, which is in good agreement with the notion that both low and high expressions of V642I-APP solely cause APO-sensitive cell death through G_0 . Therefore, it is highly likely that in addition to activation of the G_0 /NADPH oxidase pathway by low expression of N141I-PS2, high expression of N141I-PS2 generates certain additional signals that allow G_0 activation to stimulate XO.

Discussion

We have herein shown that wtPS2 weakly and N141I-PS2 strongly induce neuronal cell death, but through distinct sets of toxic mechanisms. This was somewhat unexpected, as the well known analogy with the oncogenic activation of the epidermal growth factor receptor/c-ErbB to v-ErbB prompted us to speculate that N141I-PS2-induced cytotoxicity might be based on the quantitatively amplified function of wtPS2. However, the present observation closely coincides with more

recent information that high expression of wtAPP weakly induces cell death in a manner resistant to GEE and DEVD, and low expression of both V642I-APP and NL-APP strongly induces GEE/DEVD-sensitive cytotoxicity, qualitatively different from the cytotoxicity by wtAPP (Hashimoto et al., 2000b). This study therefore indicates, for the first time, that N141I-PS2 causes GEE/DEVD-sensitive death at low expression and that cell death by high expression of N141I-PS2 is the combination of three mechanistically different cell deaths: GEE/DEVD-sensitive death (by low to high expression of N141I-PS2), GEE-sensitive/DEVD-resistant death (caused only by high expression of N141I-PS2), and GEE/DEVD-resistant death (by wtPS2).

It should be noted here that transgenic over-expression of N141I-PS2 does not result in global neuronal loss in mice (Oyama et al., 1998). Because expression of N141I-PS2 can induce cell death in isolated rodent neuronal cells—both immortalized cell lines and primary neurons (Wolozin et al., 1996; Araki et al., 2000; the present study)—a simple interpretation for this discrepancy is, as Martin and colleagues (Fukuchi et al., 1993) argued previously, that factors suppressing neurotoxicity of N141I-PS2 are present in vivo, although this hypothesis must be directly examined in transgenic models. It is also noted that the present study was performed in only one immortalized neuronal cell line. However, the principal observations that N141I-PS2 causes neuronal cell death and that PTX inhibits N141I-PS2 neurotoxicity have been reported using a different neuronal cell line (Wolozin et al., 1996) and primary cortical neurons (Araki et al., 2000), suggesting that observations noted in this study are not limited to F11/EcR cells.

The provided evidence further indicates that NADPH oxidase is involved in GEE/DEVD-sensitive cytotoxicity by low expression of N141I-PS2 and that XO is involved in GEE-sensitive/DEVD-resistant cytotoxicity by high expression of N141I-PS2, although we could not totally exclude the possibility that XO-like enzymes, such as aldehyde oxidase (Terao et al., 2000), may contribute to the latter cytotoxicity. In support of the established theory, NADPH oxidase is a major source of the oxygen-radical production not only in neutrophils but in various tissues, including neuronal cells (Noh and Koh, 2000). XO is another key enzyme in ROS formation playing a significant role in cell oxidative stress in neurons (Canas, 1999; Atlante et al., 2000). Accordingly, we have confirmed the presence of subunits of NADPH oxidase and XO in F11 cells (data not shown). Combined with the observed XO-resistant, APO/DPI-sensitive cytotoxicity by V642I-APP and with earlier studies on NL-APP-induced cytotoxicity (Hashimoto et al., 2000b), three conclusions are highly likely. First, the N141I mutation endows PS2 with the ability to trigger two distinct mechanisms for cytotoxicity, neither of which wtPS2 can activate. Second, the NADPH oxidase-mediated cytotoxic mechanism triggered by low expression of N141I-PS2 is shared with the cytotoxic mechanism by low expression of V642I-APP and NL-APP. Finally, the cytotoxic mechanism by high expression of N141I-PS2, mediated by XO, differs from that by high expression of V642I-APP and NL-APP. It has been shown that cytotoxicity by low expression of V642I-APP and NL-APP is very probably relevant to the cause of FAD (Hashimoto et al., 2000b). Therefore, NADPH oxidase-mediated GEE/DEVD-sensitive cytotoxicity by N141I-PS2, shared by both APP mutants,

would be more important as a cause of FAD than other forms of cytotoxicity.

This is also the first study describing whether and how antioxidants attenuate neurotoxicity by N141I-PS2. Guo et al. (1999) found that death-stimulating action of FAD mutant PS1 is inhibited by antioxidants. Wolozin et al. (1996) had established neurotoxicity by N141I-PS2 before neurotoxicity by FAD mutant PS1 was identified. Nonetheless, no study has so far analyzed the antioxidant action on neural death by FAD mutant PS2. The present study not only addresses this question, but indicates, in combination with our earlier study (Hashimoto et al., 2000b), that there are two different antioxidant targets, NADPH oxidase and XO, in N141I-PS2 cytotoxicity, as discussed above, and three different FAD genes (V642I-APP, NL-APP, and N141I-PS2) can induce neurotoxicity through different combinations of at least three cytotoxic mechanisms, most of which can be inhibited by antioxidants (Fig. 8).

Wolozin et al. (1996) found that PTX inhibits neurotoxicity by N141I-PS2. Yamatsuji et al. (1996) reported PTX-sensitivity of V642I-APP neurotoxicity and specified the implicated G protein to be G_o . Nonetheless, the PTX-sensitive G protein implicated in N141I-PS2 cytotoxicity remained undetermined. Using PTX-resistant mutants of G proteins, this study has identified the implicated G protein. Figure 8 summarizes the most likely mechanisms for cytotoxicity caused by N141I-PS2 and APP mutants. Low expression of N141I-PS2 induces NADPH oxidase-mediated GEE/DEVD-sensitive cytotoxicity through the PTX-sensitive G_o protein, as is the case with cytotoxicity by low expression of V642I-APP and NL-APP. Whereas low to high expression of V642I-APP solely activates this pathway and low expression of NL-APP also does so, high expression of NL-APP induces GEE/DEVD-resistant death (Hashimoto et al., 2000b). The data that G_o -mediated cytotoxicity is shared by low expression of N141I-PS2 and V642I-APP (Fig. 7A) are consistent with earlier reports that V642I-APP causes cell death through G_o , but not G_i as well as that N141I-PS2 kills neuronal cells in a PTX-sensitive manner (Wolozin et al., 1996; Yamatsuji et al., 1996; Giambarella et al., 1997).

High expression of N141I-PS2 causes XO-mediated GEE-

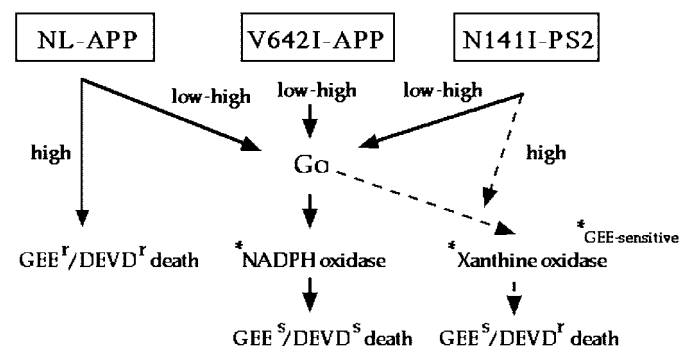


Fig. 8. Suggested mechanisms for neuronal cell death by N141I-PS2. The N141I mutation of PS2 causes GEE/DEVD-sensitive death at low expression and GEE-sensitive/DEVD-resistant death at higher expression. The former mechanism is mediated by G_o and is shared by V642I-APP (low to high expression) and NL-APP (low expression). Because the latter mechanism (GEE-sensitive/DEVD-resistant death at higher expression) is also mediated by G_o , high expression of N141I-PS2 specifically allows G_o to activate XO. In addition, high expression of V642I-APP and N141I-PS2 has an association with weak GEE-resistant/DEVD-resistant death by wtAPP and wtPS2, respectively (not shown). See the text for details.

sensitive/DEVD-resistant cytotoxicity. This pathway is again mediated by G_o , but not by G_i , and nevertheless is not activated by high expression of V642I-APP (Fig. 7B). These findings suggest that only high expression of N141I-PS2 allows activated G_o to stimulate XO. A simple interpretation would be that G_o activated by low expression of N141I-PS2—like that by low expression of V642I-APP and NL-APP—only gives rise to NADPH oxidase activation, but that highly expressed N141I-PS2 may not only activate G_o but also be able to act like a chaperone of G_o in accession to XO, allowing activated G_o to act on this enzyme. In support, chaperone-like function of PS has been postulated thus far (Gething, 2000). Alternatively, the effect of highly expressed N141I-PS2 on the action of G_o may not be direct. Investigation is necessary to clarify how N141I-PS2 regulates G_o , because this protein does not belong to a G-protein-coupled receptor group consisting of a seven-transmembrane structure. It should be noted that some proteins without such a structure, including GAP-43, the IGF-II/mannose 6-phosphate receptor, the epidermal growth factor receptor, and the transmembrane galactosyltransferase, can directly regulate the activity of specific G proteins (Murayama et al., 1990; Strittmatter et al., 1990; Gong et al., 1995; Sun et al., 1995). It should also be noted that the G_o /NADPH oxidase-mediated cytotoxicity by V642I-APP is enhanced by extracellular $A\beta$ (Hashimoto et al., 2000b). Because this pathway is shared with N141I-PS2, it is very likely that extracellular $A\beta$ potentiates the cytotoxicity by N141I-PS2, pointing to a way in which $A\beta$ contributes to neurotoxic mechanisms in AD.

Finally, it would be important that both APO and OXYP be clinically usable. Some XOIs, including OXYP, are already used clinically for patients with gout. APO, a methoxy-substituted catechol derived from the root extract of the medicinal herb *Picrorhiza kurroa*, has been shown to confer protection in animal models of arthritis and various causes of lung injury through the inhibition of NADPH oxidase in polymorphonuclear leukocytes by reacting with thiol groups required for enzyme assembly (t'Hart et al., 1991; Stolk et al., 1994; Dodd-O and Pearce, 2000). APO can be provided as a water extract. Therefore, the present study, which points to a novel possibility that the combination of APO and XO could be effective in preventing neurotoxicity by N141I-PS2, may help to open a new avenue toward the development of medical prevention for mutation-positive asymptomatic family members of this type of FAD. Considering the fact that oxidative stress could also play a toxic role in sporadic AD (Albers and Beal, 2000), the proposed combined antioxidant therapy would provide an effective method to treat at least certain factions, if not all, of sporadic AD.

Acknowledgments

We thank Dr. Mark C. Fishman for F11 neuronal hybrids; Drs. Masaki Kitajima, Sadakazu Aiso, John T. Potts Jr., and Mr. and Mrs. Y. Tamai for indispensable support; Mr. Yusuke Tomita and Dr. Yoh-ichiro Abe for cooperation; Ms. Takako Hiraki, Ms. Kazumi Nishihara, and Dr. Dovie Wylie for expert technical assistance; Drs. R. Taussig and T. Kozasa for the cDNAs encoding GaPT; Dr. M. Suematsu for indispensable cooperation and advice; and Otsuka Pharmaceutical Factory for providing (–) and (+)BOF4272. We are especially indebted to Drs. E. Garattini and T. Nishino for XO cDNA and indispensable discussion; Drs. P. St-George Hyslop and L. D'Adamio for PS2 constructs; Dr. Zongjun Shao for indispensable cooperation; and all members of the Department of Pharmacology

and Neurosciences at KEIO University School of Medicine for essential assistance.

References

- Albers DS and Beal MF (2000) Mitochondrial dysfunction and oxidative stress in aging and neurodegenerative disease. *J Neural Transm Suppl* **59**:133–154.
- Araki W, Yuasa K, Takeda S, Shirotani K, Takahashi K, and Tabira T (2000) Overexpression of presenilin-2 enhances apoptotic death of cultured cortical neurons. *Ann N Y Acad Sci* **920**:241–244.
- Atlante A, Valenti D, Gagliardi S, and Passarella S (2000) A sensitive method to assay the xanthine oxidase activity in primary cultures of cerebellar granule cells. *Brain Res Brain Res Protoc* **6**:1–5.
- Brouillet E, Trembleau A, Galanaud D, Volovitch M, Bouillot C, Valenza C, Prochiantz A, and Allinquant B (1999) The amyloid precursor protein interacts with G_o heterotrimeric protein within a cell compartment specialized in signal transduction. *J Neurosci* **19**:1717–1727.
- Canas PE (1999) The role of xanthine oxidase and the effects of antioxidants in ischemia reperfusion cell injury. *Acta Physiol Pharmacol Latinoam* **49**:13–20.
- Czech C, Lesort M, Tremp G, Terro F, Blanchard V, Schombert B, Carpentier N, Dreisler S, Bonici B, Takashima A, et al. (1998) Characterization of human presenilin 1 transgenic rats: increased sensitivity to apoptosis in primary neuronal cultures. *Neuroscience* **87**:325–336.
- Das DK, Engelman RM, Clement R, Otani H, Prasad MR, and Rao PS (1987) Role of xanthine oxidase inhibitor as free radical scavenger: a novel mechanism of action of allopurinol and oxypurinol in myocardial salvage. *Biochem Biophys Res Commun* **148**:314–319.
- Dodd-O JM and Pearce DB (2000) Effect of the NADPH oxidase inhibitor apocynin on ischemia-reperfusion lung injury. *Am J Physiol Heart Circ Physiol* **279**:H303–H312.
- Fukuchi K, Ogburn CE, Smith AC, Kunkel DD, Furlong CE, Deeb SS, Nochlin D, Sumi SM, and Martin GM (1993) Transgenic animal models for Alzheimer's disease. *Ann N Y Acad Sci* **695**:217–223.
- Gething MJ (2000) Presenilin mutants subvert chaperone function. *Nat Cell Biol* **2**:E21–23.
- Giambarella U, Yamatsuji T, Okamoto T, Matsui T, Ikezu T, Murayama Y, Levine MA, Katz A, Gautam N, and Nishimoto I (1997) G protein $\beta\gamma$ complex mediated apoptosis by familial Alzheimer's disease mutant of APP. *EMBO (Eur Mol Biol Organ) J* **16**:4897–4907.
- Gong X, Dubois DH, Miller DJ, and Shur BD (1995) Activation of a G protein complex by aggregation of β -1,4-galactosyltransferase on the surface of sperm. *Science (Wash DC)* **269**:1718–1721.
- Guo Q, Fu W, Holtsberg FW, Steiner SM, and Mattson MP (1999) Superoxide mediates the cell-death-enhancing action of presenilin-1 mutations. *J Neurosci Res* **56**:457–470.
- Guo Q, Furukawa K, Sopher BL, Pham DG, Xie J, Robinson N, Martin GM, and Mattson MP (1996) Alzheimer's PS-1 mutation perturbs calcium homeostasis and sensitizes PC12 cells to death induced by amyloid β -peptide. *Neuroreport* **8**:379–383.
- Hashimoto Y, Jiang H, Niikura T, Ito Y, Hagiwara A, Umezawa K, Abe Y, Murayama Y, and Nishimoto I (2000a) Neuronal apoptosis by apolipoprotein E4 through low-density lipoprotein receptor-related protein and heterotrimeric GTPases. *J Neurosci* **20**:8401–8409.
- Hashimoto Y, Niikura T, Ito Y, and Nishimoto I (2000b) Multiple mechanisms underlie neurotoxicity by different type Alzheimer's disease mutations of amyloid precursor protein. *J Biol Chem* **275**:34541–34551.
- Hashimoto Y, Niikura T, Ito Y, Sudo H, Hata M, Arakawa E, Abe Y, Kita Y, and Nishimoto I (2001a) Detailed characterization of neuroprotection by a rescue factor Humanin against various Alzheimer's disease-relevant insults. *J Neurosci* **21**:9235–9245.
- Hashimoto Y, Niikura T, Tajima H, Yasukawa Y, Sudo H, Ito Y, Kita Y, Kawasumi M, Kouyama K, Doyu M, et al. (2001b) A rescue factor abolishing neuronal cell death by a wide spectrum of familial Alzheimer's disease genes and $A\beta$. *Proc Natl Acad Sci USA* **98**:6336–6341.
- Luo JJ, Wallace W, Riccioni T, Ingram DK, Roth GS, and Kusiak JW (1999) Death of PC12 cells and hippocampal neurons induced by adenoviral-mediated FAD human amyloid precursor protein gene expression. *J Neurosci Res* **55**:629–642.
- Murayama Y, Okamoto T, Ogata E, Asano T, Iiri T, Katada T, Ui M, Grubb JH, Sly WS, and Nishimoto I (1990) Distinctive regulation of the functional linkage between the human cation-independent mannose 6-phosphate receptor and GTP-binding proteins by insulin-like growth factor II and mannose 6-phosphate. *J Biol Chem* **265**:17456–17462.
- Nishimoto I, Okamoto T, Matsuura Y, Takahashi S, Okamoto T, Murayama Y, and Ogata E (1993) Alzheimer amyloid protein precursor complexes with brain GTP-binding protein G_o . *Nature (Lond)* **362**:75–79.
- No D, Yao TP, and Evans RM (1996) Ecdysone-inducible gene expression in mammalian cells and transgenic mice. *Proc Natl Acad Sci USA* **93**:3346–3351.
- Noh K-M and Koh J-Y (2000) Induction and activation by zinc of NADPH oxidase in cultured cortical neurons and astrocytes. *J Neurosci* **20**:RC111.
- O'Donnell VB, Tew DG, Jones WTG, and England PJ (1993) Studies on the inhibitory mechanism of iodonium compounds with special reference to neutrophil NADPH oxidase. *Biochem J* **290**:41–49.
- Oyama F, Sawamura N, Kobayashi K, Morishima-Kawashima M, Kuramochi T, Ito M, Tomita T, Maruyama K, Saido TC, Iwatsubo T, et al. (1998) Mutant presenilin 2 transgenic mouse: effect on an age-dependent increase of amyloid β -protein 42 in the brain. *J Neurochem* **71**:313–322.
- Shastri BS and Giblin FJ (1999) Genes and susceptible loci of Alzheimer's disease. *Brain Res Bull* **48**:121–127.
- Stolk J, Hiltermann TJN, Dijkman JH, and Verhoeven AJ (1994) Characteristics of

- the inhibition of NADPH oxidase activation in neutrophils by apocynin, a methoxy-substituted catechol. *Am J Respir Cell Mol Biol* **11**:95–102.
- Strittmatter SM, Valenzuela D, Kennedy TE, Neer EJ, and Fishman MC (1990) G_o is a major growth cone protein subject to regulation by GAP-43. *Nature (Lond)* **344**:836–841.
- Sudo H, Jiang H, Yasukawa T, Hashimoto Y, Niikura T, Kawasumi M, Matsuda S, Takeuchi Y, Aiso S, Matsuo M, et al. (2000) Antibody-regulated neurotoxic function of cell-surface β -amyloid precursor protein. *Mol Cell Neurosci* **16**:708–723.
- Sun H, Seyer JM, and Patel TB (1995) A region in the cytosolic domain of the epidermal growth factor receptor antithetically regulates the stimulatory and inhibitory guanine nucleotide-binding regulatory proteins of adenylyl cyclase. *Proc Natl Acad Sci USA* **92**:2229–2233.
- Suzuki H, DeLano FA, Parks DA, Jamshidi N, Granger DN, Ishii H, Suematsu M, Zweifach BW, and Schmid-Schonbein GW (1998) Xanthine oxidase activity associated with arterial blood pressure in spontaneously hypertensive rats. *Proc Natl Acad Sci USA* **95**:4754–4759.
- Taussig R, Sanchez S, Rifo M, Gilman AG, and Belardetti F (1992) Inhibition of the ω -conotoxin-sensitive calcium current by distinct G proteins. *Neuron* **8**:799–809.
- Terao M, Kurosaki M, Saltini G, Demontis S, Marini M, Salmona M, and Garattini E (2000) Cloning of the cDNAs coding for two novel molybdo-flavoproteins showing high similarity with aldehyde oxidase and xanthine oxidoreductase. *J Biol Chem* **275**:30690–30700.
- t'Hart BA, Bakker NP, Labadie RP, and Simons JM (1991) The newly developed neutrophil oxidative burst antagonist apocynin inhibits joint-swelling in rat collagen arthritis. *Agents Actions Suppl* **32**:179–184.
- t'Hart BA and Simons JM (1992) Metabolic activation of phenols by stimulated neutrophils: a concept for a selective type of anti-inflammatory drug. *Biotechnol Ther* **3**:119–135.
- Vito P, Ghayur T, and D'Adamio L (1997) Generation of anti-apoptotic presenilin-2 polypeptides by alternative transcription, proteolysis, and caspase-3 cleavage. *J Biol Chem* **272**:28315–28320.
- Weihl CC, Ghadge GD, Kennedy SG, Hay N, Miller RJ, and Roos RP (1999) Mutant presenilin-1 induces apoptosis and downregulates Akt/PKB. *J Neurosci* **19**:5360–5369.
- Wolozin B, Iwasaki K, Vito P, Ganjei JK, Lacana E, Sunderland T, Zhao B, Kusiak JW, Wasco W, and D'Adamio L (1996) Participation of presenilin 2 in apoptosis: enhanced basal activity conferred by an Alzheimer mutation. *Science (Wash DC)* **274**:1710–1713.
- Yamatsuji T, Okamoto T, Takeda S, Fukumoto H, Iwatsubo T, Suzuki N, Asami-Odaka A, Ireland S, Kinane TB, and Nishimoto I (1996) G protein-mediated neuronal DNA fragmentation induced by familial Alzheimer's disease-associated mutants of APP. *Science (Wash DC)* **272**:1349–1352.
- Zhao B, Chrest FJ, Horton WE Jr, Sisodia SS, and Kusiak JW (1997) Expression of mutant amyloid precursor proteins induces apoptosis in PC12 cells. *J Neurosci Res* **47**:253–263.

Address correspondence to: Dr. Ikuro Nishimoto, Department of Pharmacology and Neurosciences, KEIO University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. E-mail: nisimoto@sc.itc.keio.ac.jp
