Secreted β-Amyloid Precursor Protein Counteracts the Proapoptotic Action of Mutant Presenilin-1 by Activation of NF-κB and Stabilization of Calcium Homeostasis*

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Mutations in the presenilin-1 (PS-1) gene account for approximately 50% of the cases of autosomal dominant, early onset, inherited forms of Alzheimer's disease (AD). PS-1 is an integral membrane protein expressed in neurons and is localized primarily in the endoplasmic reticulum (ER). PS-1 mutations may promote neuronal degeneration by altering the processing of the β-amyloid precursor protein (APP) and/or by engaging apoptotic pathways. Alternative processing of APP in AD may increase production of neurotoxic amyloid β-peptide (Aβ) and reduce production of the neuroprotective α-secretase-derived form of APP (sAPPα). In differentiated PC12 cells expressing an AD-linked PS-1 mutant (L286V), sAPPα activated the transcription factor NF-κB and prevented apoptosis induced by Aβ. Treatment of cells with αβ decoy DNA blocked the antiapoptotic action of sAPPα, demonstrating the requirement for NF-κB activation in the cytoprotective action of sAPPα. Cells expressing mutant PS-1 exhibited an aberrant pattern of NF-κB activity following exposure to Aβ, which was characterized by enhanced early activation of NF-κB followed by a prolonged depression of activity. Blockade of NF-κB activity in cells expressing mutant PS-1 by αβ decoy DNA was associated with enhanced Aβ-induced increases of [Ca2+]i, and mitochondrial dysfunction. Treatment of cells with sAPPα stabilized [Ca2+]i and mitochondrial function and suppressed oxidative stress by a mechanism involving activation of NF-κB. Blockade of ER calcium release prevented (and stimulation of ER calcium release by thapsigargin induced) apoptosis in cells expressing mutant PS-1, suggesting a pivotal role for ER calcium release in the proapoptotic action of mutant PS-1. Finally, a role for NF-κB in preventing apoptosis induced by ER calcium release was demonstrated by data showing that sAPPα prevents thapsigargin-induced apoptosis, an effect blocked by αβ decoy DNA. We conclude that sAPPα stabilizes cellular calcium homeostasis and protects neuronal cells against the proapoptotic action of mutant PS-1 by a mechanism involving activation of NF-κB. The data further suggest that PS-1 mutations result in aberrant NF-κB regulation that may render neurons vulnerable to apoptosis.

Alzheimer’s disease (AD)1 is characterized by the accumulation of amyloid β-peptide (Aβ) and death of neurons in brain regions involved in learning and memory processes (1). Neuro- nal apoptosis (2, 3) is implicated in AD based on studies of postmortem brain tissues (4, 5) and cell culture studies showing that Aβ can induce apoptosis (6, 7). Some cases of AD are inherited in an autosomal dominant manner and are characterized by early age of onset; approximately half of these cases are caused by mutations in the presenilin-1 (PS-1) gene located on chromosome 14 (8–10). PS-1 encodes an integral membrane protein with six or eight membrane-spanning domains (11–13) and is localized in the endoplasmic reticulum (ER; Refs. 14–17). PS-1 is expressed in neurons throughout the brain (18–21) and is present in both degenerating and nondegenerating neurons in AD brain (22, 23). Consequences of PS-1 and PS-2 mutation expression in cultured cells include increased production of a long form of Aβ (Aβ41–42); Refs. 24 and 25), decreased choline acetyltransferase activity (26), and increased vulnerability of cells to apoptosis (27–30). The mechanism(s) whereby PS mutations sensitize neurons to apoptosis is unknown.

Links between aberrant proteolytic processing of APP and neuronal degeneration in AD are supported by considerable data (for a review, see Ref. 31). APP mutations, which account for some cases of inherited AD, may simultaneously increase production of neurotoxic forms of Aβ and decrease production of neuroprotective secreted forms of APP (sAPPα). Aβ can induce neuronal apoptosis and can increase neuronal vulnerability to excitotoxicity by a mechanism involving induction of oxidative stress (32–36) and disruption of calcium homeostasis (35–39). On the other hand, sAPPα stabilizes neuronal calcium homeostasis and protects neurons against excitotoxic, metabolic, and oxidative insults including exposure to Aβ (40, 41).

It was recently reported that sAPPα induces activation of the transcription factor NF-κB in cultured hippocampal neurons (42). NF-κB exists in the cytosol as an inducible three-subunit complex consisting of the transcription factor dimer (p50 and p65) and an associated inhibitory subunit called I-κB (43). NF-κB activation occurs when I-κB is induced to dissociate from the complex, a process that may involve phosphorylation, proteolysis, and/or oxidative damage to I-κB. Recent findings suggest that NF-κB plays an antiapoptotic role in nonneuronal cells (44, 45) and in neurons (46–48). Agents that activate NF-κB (e.g. tumor necrosis factor and ceramide) can prevent cell death induced by excitotoxic, metabolic, and oxidative insults including exposure to Aβ (49, 50), whereas αβ decoy DNA...
Secreted APP, Presenilins, and NF-κB

FIG. 1. Secreted APPα protects PC12 cells against the proapoptotic action of mutant PS-1: evidence for the involvement of NF-κB activation and stabilization of calcium homeostasis. The indicated lines of PC12 cells were pretreated for 24 h with sAPPα (10 nM) or κB decoy DNA (25 μM), or for 1 h with dantrolene (DTL; 10 μM) or nifedipine (Nif; 1 μM) and were then exposed to Aβ (50 μM) for 48 h, and apoptosis was quantified (see “Materials and Methods”). PC12 cell lines analyzed included untransfected cells (A), vector-transfected cells (B), two different lines overexpressing wild-type PS-1 (C and D), and two different lines overexpressing mutant PS-1 (E and F). Values are the mean and S.E. of determinations made in 4–6 separate cultures. *, p < 0.01 compared with control and sAPPα values. **, p < 0.01 compared with the values for cultures exposed to Aβ, Aβ plus decoy DNA, and sAPPα plus Aβ plus decoy DNA. For panels A–D the value for cells exposed to Aβ plus decoy DNA was significantly greater than the value for cultures exposed to Aβ alone (p < 0.05). Values for PS-1L286VC1 and PS-1L286VC9 lines exposed to Aβ, Aβ plus decoy DNA, and Aβ plus sAPPα plus decoy DNA were significantly greater than corresponding values in each of the other cell lines (p < 0.05) (ANOVA with Scheffe’s post hoc tests).

MATERIALS AND METHODS

PC12 Cell Lines and Experimental Treatments—Rat pheochromocytoma (PC12) cell lines stably expressing human wild-type PS-1 and mutant PS-1 (L286V) were established using the “tet-off” expression system (Promega), where the expression of the transgene is under the control of a tetracycline-sensitive transactivator, as described in detail previously (40). Purity was confirmed by Western blot analysis and silver staining after SDS-polyacrylamide gel electrophoresis. Double-stranded κB decoy DNA was prepared by annealing complementary single strands (2 μM each) with the sequences 5′-GAGGGGACTTTC-3′ and 5′-AGGGGAAAGTCCCCT-3′. Nifedipine, sodium dantrolene, and thapsigargin were purchased from Sigma and prepared as 9 M stocks in ethanol.

Quantification of Apoptosis and Mitochondrial Function—Methods for analysis of apoptosis are detailed in our previous studies (28, 36). Briefly, cells were stained with the fluorescent DNA-binding dyes Hoechst 33342 or propidium iodide, and cells were visualized under epifluorescence illumination. Cells with condensed and fragmented (apoptotic) nuclei were counted in four random × 40 fields per culture; counts were made without knowledge of cell line or treatment history. Images of propidium iodide-stained cells were acquired with a confocal

(which blocks NF-κB activity) enhances cell death in several paradigms (47, 48). NF-κB activity may be altered in vulnerable brain regions in AD patients, such that its activity is increased in neurons associated with plaque amyloid (51, 52). We now report that sAPPα counteracts the proapoptotic actions of mutant PS-1 by activating NF-κB and stabilizing calcium homeostasis. NF-κB appears to interrupt the apoptotic program at an early stage prior to oxyradical production and mitochondrial dysfunction. An abnormal sustained suppression of NF-κB activity following exposure of cells to Aβ occurred in cells overexpressing mutant (but not wild-type) PS-1, suggesting a role for this transcription factor in the pathogenic mechanism of PS-1 mutations.

PC12 cell lines were maintained at 37 °C (5% CO2 atmosphere) in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum. For differentiation, cells were maintained in the presence of 50 ng/ml nerve growth factor for 14 days in RPMI containing 2% bovine serum albumin (which blocks NF-κB activity) and 10 μM tetracycline; the tetracycline was switched to Locke’s solution, which contained 154 mM NaCl; 5.6 mM KCl, 2.3 mM CaCl2, 1.0 mM MgCl2, 3.6 mM NaHCO3, 10 mM glucose, 5 mM Hepes buffer (pH 7.2). Secreted APPα (sAPPα955) was purified from the culture supernatant of human embryonic kidney 293 cells transfected with the corresponding cDNA constructs as described previously (40). Purity was confirmed by Western blot analysis and silver staining after SDS-polyacrylamide gel electrophoresis. Double-stranded κB decoy DNA was prepared by annealing complementary single strands (2 μM each) with the sequences 5′-GAGGGGACTTTC-3′ and 5′-AGGGGAAAGTCCCCT-3′. Nifedipine, sodium dantrolene, and thapsigargin were purchased from Sigma and prepared as 9 M stocks in ethanol.
laser scanning microscope (Molecular Dynamics; 488-nm excitation and 510-nm barrier filter) using a × 60 oil immersion objective. Two methods were employed to assess mitochondrial function. Mitochondrial transmembrane potential was assessed using the dye rhodamine 123 (Molecular Probes, Inc.) as described previously (33). Cultures were incubated for 30 min in RPMI 1640, containing 5 μM rhodamine 123, and were then washed with Locke’s solution. Cellular fluorescence was imaged using a laser scanning confocal microscope with excitation at 488 nm and emission at 510 nm. Levels of cellular MTT reduction, a measure of mitochondrial energy charge/redox state (54), were quantified as described previously (36). Briefly, MTT solution (5 mg/ml phosphothionate and sodium) was added to cultures (1:10, MTT solution:cell culture medium, v/v) and allowed to incubate for 3 h. The cells were washed three times with Locke’s solution and solubilized in dimethyl sulfoxide, and the absorbance (592 nm) in each well was quantified using a plate reader.

Measurements of [Ca2+]i and Cellular Perioxidase Levels—Fluorescence ratio imaging of the calcium indicator dye fura-2 was performed as described previously (37, 38). Briefly, following exposure to Aβ, cells were loaded with fura-2 (30-min incubation in the presence of 10 μM fura-2), and maintained in the presence of Aβ and other treatments during imaging. Fluorescence was imaged using a Zeiss AttoFluor system with a × 40 oil objective; the average [Ca2+]i, in individual neuronal cell bodies was determined from the ratio of the fluorescence emissions obtained from two different excitation wavelengths (334 and 380 nm). The system was calibrated using solutions containing either no Ca2+ or a saturating level of Ca2+ (1 mM) using the formula [Ca2+]=[Kd(R-Rm)/(Rm-R)](Fs/Fp). Relative levels of cellular peroxides were quantified in individual PC12 cells by confocal laser scanning microscope analysis using the dye 2′,7′-dichlorofluorescin diacetate (Molecular Probes) as described previously (33). Briefly, cells were incubated for 50 min in the presence of 50 μM dye and then washed three times (2 ml/wash) in Locke’s solution. Images of cellular fluorescence were acquired using a confocal laser scanning microscope (Molecular Dynamics) with excitation at 488 nm and emission at 510 nm. The intensity of the laser beam and the sensitivity of the photodetector were held constant to allow quantitative comparisons of relative fluorescence intensity of cells between treatment groups. Cells were located under visible light and scanned only once with the laser to avoid artifacts associated with photo-oxidation. Fluorescence intensities in cell bodies were quantified using Molecular Dynamics “ImageSpace” software.

Electrophoretic Mobility Gel Shift Assay—These methods were similar to those described previously (46, 48). Briefly, cell extracts containing DNA-binding proteins were prepared, and gel shift assays were performed using a commercially available assay kit (Promega). Double-stranded, 32P-labeled DNA (κB consensus sequence 5′-AGT TGA GGG TAT CCC AGG C-3′; 100,000 cpm) was added to a reaction mixture containing 5 μl nuclelease-free water, 2 μl of gel shift binding 5× buffer (20% glycerol, 5 mM MgCl2, 2.5 mM EDTA, 2.5 mM diithiothreitol, 250 mM NaCl, 0.25 mg/ml poly[d(I-C)poly[d(I-C)]], and 50 μM Tris-HCl, pH 7.5) and 2 μl (5 μg) of cell extract. The reaction was allowed to proceed for 30 min at room temperature, and then 1 μl of gel loading 10× buffer (40% glycerol, 0.2% bromphenol blue, 250 μM Tris-HCl, pH 7.5) was added. Samples were separated by electrophoresis through a non-denaturing 4% acrylamide gel, and the gel was dried and exposed to x-ray film. To demonstrate the specificity of the inducible NF-κB complexes in PC12 cells overexpressing PS-1 mutation detected in gel shift reactions, we performed five reactions: a positive control (where the 32P-labeled specific NF-κB consensus oligonucleotide was included in the binding reaction), a negative control (where no DNA-binding protein extract was added in the binding reaction), a specific competition assay (where an excess of unlabeled specific competitor NF-κB oligonucleotide probe was included in the binding reaction), a mutant κB competition assay (where an excess of unlabeled mutant NF-κB oligonucleotide 5′-GAT CGA ACT GAC CCC CGG CCC GT-3′ was included in the binding reaction). To further determine the specificity of the gel shift reaction and to examine the possible subunit composition of the inducible NF-κB complexes, we performed supershift experiments with antibody raised against p50 or p65 proteins purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-p50 antibody was rabbit IgG raised against an epitope corresponding to amino acids 350–363 within the nuclear localization signal region of human p50. The anti-p65 antibody was rabbit IgG raised against the N-terminal amino acids 3–19 of the human p65. As a control for p50 and p65 antibodies, extracts of DNA-binding proteins were also incubated in the presence of preimmune rabbit serum. Supershift experiments were performed by adding 1.0 μl of the antibody per 10 μl of reaction volume prior to the addition of 32P-labeled oligonucleotide probe, and the mixtures were incubated overnight at 4 °C. Relative levels of DNA-protein hybrids were quantified by densitometric analysis of scanned gel images using NIH Image 1.47 software.

RESULTS

sAPPα Protects PC12 Cells Expressing Mutant PS-1 against Apoptosis Induced by Aβ—We examined the effects of mutant PS-1 and sAPPα on vulnerability of PC12 cells to apoptosis induced by Aβ (Aβ(1–42)) using two cell lines overexpressing wild-type human PS-1 (PS-1C3 and PS-1C7), two lines expressing the L286V PS-1 mutation (PS-1L286VC1 and PS-1L286VC9), a vector-transfected control line, and the untransfected parent cell line. Preliminary Western blot analysis showed that levels of overexpression of wild-type and mutant PS-1 48 h following withdrawal of tetracycline were similar in the selected cell lines under the conditions employed (data not shown; cf. Ref. 28). Basal levels of apoptosis in the various cell lines ranged from 1 to 4% (Fig. 1). In previous concentration-effect studies, we found that an Aβ(1–42) concentration of 50 μM reliably induced a moderate level of apoptosis in untransfected PC12 cells during 24–48-h exposure periods (28, 36). Exposure to 50 μM Aβ for 48 h induced apoptosis in 30–40% of cells in control PC12 cell lines and lines expressing wild-type PS-1; apoptosis was significantly enhanced (to 60–70%) in lines expressing mutant PS-1 (Figs. 1 and 2). Mutations in APP responsible for some inherited forms of AD may promote neuronal degeneration by increasing levels of neurototoxic Aβ and/or by decreasing production of sAPPα, which has been shown to protect neurons against excitotoxic and oxidative insults (for a review, see Ref. 31). When cell lines were pretreated for 24 h with 10 nM sAPPα, there was a highly significant suppression of Aβ-induced apoptosis in both control lines and lines expressing mutant PS-1 (Fig. 1).

Involvement of NF-κB in the Antiapoptotic Action of sAPPα—A variety of intercellular signals that can prevent cell death induce activation of the transcription factor NF-κB in neural cells including tumor necrosis factor-α (TNFα) (46), neural growth factor (47), and sAPPα (42). NF-κB is also activated in cells exposed to a variety of insults, including Aβ, that
FIG. 3. Effects of mutant PS-1 and sAPPα on NF-κB activity following exposure of PC12 cells to Aβ. A, PC12 cells were exposed for 24 h to vehicle (Control) or 25 μM κB decoy DNA. Cell extracts were subjected to gel shift analysis. Specificity of the protein-DNA interactions representing the shifted bands was established in samples in which excess cold competitor DNA (lane 4) or nonspecific (scrambled) DNA (lane 2).
induce oxidative stress and increase [Ca\(^{2+}\)](32, 52), which may represent a stress response designed to protect the cells. In order to determine whether NF-κB activation mediated the antiapoptotic action of sAPPα in PC12 cells, we employed κB decoy DNA to block activation of NF-κB (48, 55). Whereas κB decoy DNA alone had no effect on basal levels of apoptosis (data not shown), it significantly enhanced Aβ-induced apoptosis and completely abolished the antiapoptotic effect of sAPPα (Fig. 1).

We next performed gel shift analysis to measure levels of NF-κB activity following exposure to Aβ and sAPPα in the different PC12 cell lines. In agreement with a previous study (56), we observed two shifted bands representing κB-binding proteins in unstimulated PC12 cells (Fig. 3A). The intensity of the bands was also markedly reduced in cultures treated with 25 μM κB decoy DNA (Fig. 3A). Gel shift analysis showed that both Aβ and sAPPα induced NF-κB activity and that κB decoy DNA prevented activation of NF-κB by each agent (Figs. 3, B–D, and 4). The two shifted bands were specific κB-binding proteins, since they were eliminated by incubation with excess cold specific κB competitor DNA but not by cold mutant κB or nonspecific (AP2) competitor oligonucleotide (Fig. 3E). Super-shift analysis demonstrated that the higher molecular weight band of NF-κB was supershifted by the p65 antibody, while the lower molecular weight band of NF-κB was supershifted by the p50 antibody, indicating that the higher molecular weight band was p65, while the lower molecular weight band was p50 (Fig. 3E).

We next examined the pattern of NF-κB activation in response to Aβ in control PC12 cell lines and lines expressing mutant PS-1. Control cell lines and lines expressing mutant PS-1 were exposed to Aβ at different time periods, and levels of NF-κB activity were quantified by gel shift analysis. Marked, but transient, increases in NF-κB activity occurred within 4 h of exposure to Aβ in each cell line; levels of DNA binding activity of each of the two different bands were increased in cells exposed to Aβ (Figs. 3 and 4). The magnitude of the early NF-κB induction was greater in lines expressing mutant PS-1 than in control lines, a result consistent with increased levels of oxidative stress and [Ca\(^{2+}\)](32, 52) in cells expressing mutant PS-1 (see below). However, levels of NF-κB activity decreased progressively 8, 24, 48, and 72 h following exposure to Aβ in all cell lines. Notably, levels of NF-κB activity 24–48 h following exposure to Aβ were significantly lower in cells expressing mutant PS-1 compared with control cell lines (Figs. 3 and 4). There was a correlation between a very low level of NF-κB activity beginning at 8 h after Aβ treatment in the lower shifted band and 24 h post-Aβ in the upper shifted band and increased apoptosis in cells expressing mutant PS-1 (Figs. 1 and 4). Treatment of cells expressing mutant PS-1 with sAPPα prevented the prolonged suppression of NF-κB activity that occurred 24–48 h following exposure to Aβ in the absence of sAPPα, and, in fact, levels of NF-κB activity remained higher than in unstimulated control cultures (Figs. 3D and 4). Collectively, these findings suggest a role for sustained increases in NF-κB activity in the cytoprotective action of sAPPα.

**Cellular Calcium Homeostasis Is Disrupted by Mutant PS-1 and Restored by sAPPα—**Because elevations of [Ca\(^{2+}\)](32, 37, 38) and because sAPPα has been shown to stabilize [Ca\(^{2+}\)](32, 52), we observed two shifted bands representing κB-binding proteins in unstimulated PC12 cells (Fig. 3A). The intensity of the bands was also markedly reduced in cultures treated with 25 μM κB decoy DNA (Fig. 3A). Gel shift analysis showed that both Aβ and sAPPα induced NF-κB activity and that κB decoy DNA prevented activation of NF-κB by each agent (Figs. 3, B–D, and 4). The two shifted bands were specific κB-binding proteins, since they were eliminated by incubation with excess cold specific κB competitor DNA but not by cold mutant κB or nonspecific (AP2) competitor oligonucleotide (Fig. 3E). Super-shift analysis demonstrated that the higher molecular weight band of NF-κB was supershifted by the p65 antibody, while the lower molecular weight band of NF-κB was supershifted by the p50 antibody, indicating that the higher molecular weight band was p65, while the lower molecular weight band was p50 (Fig. 3E).

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Fig. 4. Densitometric analysis of κB DNA-binding activities corresponding to the upper shifted band (A and B) and the lower shifted band (C and D) (see Fig. 3). The time courses of changes in levels of each active κB-binding protein following exposure to 50 μM Aβ in control cultures (A and C) and in cultures pretreated for 24 h with 10 nM sAPPα (B and D) are shown. Values are expressed as a percentage of the level in untreated control cultures and represent the mean and S.E. of determination made in three or four separate experiments. The values for PC12 cells expressing mutant PS-1 were significantly different from the values for each of the other cell lines as follows. A, 4 h (p < 0.01), 8 h (p < 0.05), 24 h (p < 0.01), and 48 h (p < 0.05); B, 4 h (p < 0.01); C, 4 h (p < 0.01), 24 h (p < 0.01), 48 h (p < 0.01); D, 4 h, p < 0.01, 24 h (p < 0.05) (ANOVA with Scheffe’s post hoc tests). Overexpression of either wild-type or mutant PS-1 did not affect basal NF-κB activity under normal culture conditions.

paradigms (57, 58). Because PS-1 is located in the ER, and because dantrolene protected PC12 cells against the proapoptotic action of mutant PS-1 (Fig. 1), we determined whether sAPPα would protect cells against apoptosis induced by thapsigargin, an agent known to induce apoptosis by selectively inhibiting the ER Ca\(^{2+}\)-ATPase (59). Pretreatment with sAPPα resulted in a significant reduction in apoptosis induced by thapsigargin in each control cell line and in lines expressing mutant PS-1 (Fig. 6). The protective effect of sAPPα against thapsigargin-induced apoptosis was abolished in cultures cotreated with κB decoy DNA (data not shown) indicating a necessary role for NF-κB activation in suppression of apoptosis induced by ER calcium release.

Enhanced Mitochondrial Dysfunction and Oxidative Stress in PC12 Cells Expressing Mutant PS-1: Protection by sAPPα—Mitochondrial alterations, including a decrease in mitochondrial transmembrane potential and energy charge/redox state, have been identified as being central to the effector phase of apoptosis (3) and may contribute to the neurotoxic action of Aβ (36, 54). Exposure of PC12 cells to Aβ for 4 h resulted in decreases in levels of mitochondrial membrane potential, as quantified using the fluorescent probe rhodamine 123 (Fig. 7Aa). The decrease in rhodamine 123 fluorescence was significantly greater in cells expressing mutant PS-1 compared with control cell lines and lines overexpressing wild-type PS-1 (Fig. 7Aa). Pretreatment of cultures with sAPPα prior to exposure to Aβ resulted in a significant attenuation of the decrease in transmembrane potential, which was particularly pronounced in cell lines expressing mutant PS-1 (Fig. 7, A and C). κB decoy DNA suppressed the ability of sAPPα to prevent Aβ-induced decrease in rhodamine 123 fluorescence (Fig. 7Aa) and enhanced the decrease in rhodamine 123 fluorescence induced by Aβ. Exposure of the different cell lines to κB decoy DNA alone caused no change in rhodamine 123 fluorescence during exposures of up to 12 h (Fig. 7Ba). However, 24 h following exposure to κB decoy DNA, there was a significant decrease in mitochondrial transmembrane potential in cells expressing mutant PS-1 but not in control cell lines and lines overexpressing wild-type PS-1 (Fig. 7Ba). The latter result suggests that basal levels of NF-κB activity play a role in counteracting an adverse effect of mutant PS-1 on mitochondrial function. In parallel experiments, we quantified levels of MTT reduction, a measure of mitochondrial energy charge/redox state (54), in the different cell lines following exposure to Aβ in the presence or absence of sAPPα and/or κB decoy DNA. Exposure of PC12 cells to Aβ for 4 h resulted in a decrease in the level of MTT reduction that was significantly greater in cells expressing mutant PS-1 compared with control cell lines; pretreatment with sAPPα resulted in a significant attenuation of Aβ-induced decrease in MTT reduction, and this action of sAPPα was largely blocked by κB decoy DNA (data not shown).

Previous studies showed that Aβ induces oxidative stress in cultured neurons (32–36) and that this action of Aβ is enhanced in PC12 cells expressing mutant PS-1 (28). In order to determine whether sAPPα modifies levels of oxidative stress and if NF-κB is involved, we measured relative levels of cellular peroxides using the fluorescent probe DCF (33). Exposure of PC12 cells to Aβ for 4 h resulted in an increase in DCF fluorescence that was significantly greater in cells expressing mutant PS-1 compared with control cell lines and lines overexpressing wild-type PS-1 (Fig. 8Aa). The Aβ-induced increase in DCF fluorescence was blocked in cells pretreated with sAPPα (Fig. 8a). No suppression of Aβ-induced DCF fluorescence occurred in cultures cotreated with κB decoy DNA and sAPPα (data not shown).

**DISCUSSION**

The present findings demonstrate that sAPPα can protect PC12 cells against the proapoptotic action of mutant PS-1 by a mechanism involving activation of NF-κB, stabilization of calcium homeostasis and mitochondrial function, and suppression of oxidative stress. Recent studies of tumor cells (44, 45, 60) and cultured embryonic rat hippocampal neurons (48) have shown that activation of NF-κB (in response to TNFα) can prevent apoptosis induced by several different insults including exposure to oxidative insults. We found that sAPPα induces NF-κB activation in PC12 cells and protects them from being killed by Aβ. The cytoprotective effect of sAPPα was largely prevented by co-treatment with κB decoy DNA, a result that provides evidence for a cause-effect relationship between acti-
viation of NF-κB and prevention of apoptosis. The enhancement of Aβ-induced apoptosis in PC12 cells overexpressing mutant human PS-1 was abrogated in cultures pretreated with sAPPα, an effect blocked by αB-decoy DNA. Previous studies have shown that the mechanism whereby Aβ damages and kills neurons involves induction of oxidative stress, disruption of calcium homeostasis, and mitochondrial dysfunction (for a review, see Ref. 31). We found that Aβ caused increases of [Ca²⁺]i and peroxide levels, and mitochondrial transmembrane potential at nearly normal levels following exposure to Aβ. The ability of αB decoy DNA to counteract the stabilizing effect of sAPPα on [Ca²⁺]i, peroxide levels, and mitochondrial transmembrane potential indicates a central role for NF-κB in the antiapoptotic mechanism of action of sAPPα.

Gel shift analyses of NF-κB activation following exposure of PC12 cells to Aβ revealed differences in the pattern of NF-κB activity in cells overexpressing mutant PS-1 compared with cells overexpressing wild-type PS-1 and control cell lines. A striking consequence of mutant PS-1 expression was a sustained suppression of NF-κB activity, which was evident as early as 8 h following exposure of cells to Aβ, which was correlated with increased apoptosis measured 48 h following exposure to Aβ. In light of recent data showing that activation of NF-κB in cells exposed to apoptotic stimuli suppresses cell death (44–46, 48, 60), our findings suggest that the sustained suppression of NF-κB activity plays a role in the proapoptotic action of mutant PS-1. However, because the apoptotic process was enhanced in cells expressing mutant PS-1, we cannot completely rule out the possibility that the depressed NF-κB activity in cells expressing mutant PS-1 was a consequence of early events in the apoptotic process. Nevertheless, the ability of sAPPα to prevent both the delayed suppression of NF-κB activity and apoptosis in cells expressing mutant PS-1 and the ability of αB decoy DNA to block these effects of sAPPα support a role for suppression of NF-κB activity in the pathogenic mechanism of PS-1 mutations. The enhanced activation of NF-κB during the first 4 h following exposure to Aβ in cells expressing mutant PS-1 probably reflects the enhanced [Ca²⁺]i, and oxidative stress documented in our calcium imaging and DCF fluorescence assays. Indeed, both calcium and hydrogen peroxide are known to be potent activators of NF-κB in many cell types including neurons (61–63). When taken together with the data showing that αB decoy DNA enhanced Aβ-induced cell death, the enhanced short term activation of NF-κB in PC12 cells expressing mutant PS-1 is clearly not involved in the enhanced vulnerability of these cells to apoptosis induced by Aβ and may represent a self-defense mechanism of the cells.

The gene targets of NF-κB that mediate cellular resistance to apoptosis have not been firmly established. However, two candidates are Mn-superoxide dismutase and the calcium-binding protein calbindin D28k. Both Mn-superoxide dismutase and calbindin D28k are induced by TNFα and ceramide in cultured...
embryonic rat hippocampal neurons (48, 49), and overexpression of calbindin D28k (64) and Mn-superoxide dismutase (65) in cultured neurons protects them from being killed by oxidative and metabolic apoptotic insults. Moreover, it was recently shown that \( \kappa \)-B decoy DNA blocks both induction of Mn-superoxide dismutase expression and resistance to apoptosis in hippocampal neurons treated with TNF\( \alpha \) (48), strongly suggesting a role for this antioxidant enzyme in the neuroprotective actions of NF-\( \kappa \)-B activation. The evidence that A\( \beta \) toxicity in neurons involves both oxidative stress and elevation of \([\text{Ca}^{2+}]_i\) (31) is consistent with a role for NF-\( \kappa \)-B-mediated regulation of expression of genes involved in regulating calcium homeostasis and free radical metabolism in the cytoprotective actions of sAPP\( \alpha \) documented in the present study.
The ability of dantrolene and nifedipine to protect PC12 cells against the proapoptotic action of mutant PS-1 suggests pivotal roles of calcium release from ER and influx through voltage-dependent channels in the pathogenic mechanism of PS-1 mutations. Consistent with this interpretation are recent data showing that overexpression of the antiapoptotic gene product Bcl-2 stabilizes [Ca\(^{2+}\)]\(_i\) in PC12 cells expressing mutant PS-1. Cultures were pretreated for 24 h with 0.01% saline or 1% sAPP\(\alpha\) and were then exposed to either 0.5% water (control and sAPP\(\alpha\)) or 50 nM A\(\beta\) for 4 h. Relative levels of DCF fluorescence were indicated by the scale bar (lower right). Note that A\(\beta\) caused a marked increase in levels of DCF fluorescence, which was much less pronounced in cells pretreated with sAPP\(\alpha\).

Our data therefore suggest that aberrant ER calcium regulation plays a central role in A\(\beta\)-induced apoptosis and that PS-1 mutations may promote apoptosis by enhancing ER calcium release. sAPP\(\alpha\) stabilized [Ca\(^{2+}\)]\(_i\), and suppressed A\(\beta\)- and thapsigargin-induced apoptosis in cells expressing mutant PS-1. Stabilization of calcium homeostasis in cells treated with sAPP\(\alpha\) was correlated with maintenance of NFKB activity and mitochondrial function, consistent with the ability of well-known activators of NF-\(\kappa\)B such as TNF\(\alpha\) to stabilize [Ca\(^{2+}\)]\(_i\) (46, 48).

Studies of brain tissue and cultured fibroblasts from AD patients have provided evidence for perturbed calcium homeostasis in AD. Examples include the following: 1) calcium-dependent protease activity is increased in degenerating neurons in AD brain tissue (68); 2) levels of acylphosphatase, an enzyme that modulates the activity of the ER Ca\(^{2+}\)-ATPase, are in-

\[^{2}\]Q. Guo and M. P. Mattson, unpublished data.
increased in fibroblasts from patients bearing PS-1 mutations (67); 3) Ca\(^{2+}\) release from ER in response to agonists linked to the inositol 1,4,5-trisphosphate pathway is increased in fibroblasts from carriers of PS-1 mutations (68); and 4) levels of inositol 1,4,5-trisphosphate binding to ER membranes were reported to be decreased in cerebral cortical tissue from sporadic AD patients (69). When taken together with data showing altered levels of NF-\(\kappa\)B immunoreactivity and activity in neurons in vulnerable regions of AD patients (50, 51, 70), our findings suggest mechanistic links between disruption of ER calcium homeostasis, NF-\(\kappa\)B activity, and neuronal apoptosis in AD. The present findings also identify systems regulating ER calcium homeostasis (e.g., inositol 1,4,5-trisphosphate receptors and Ca\(^{2+}\)-ATPases), NF-\(\kappa\)B activity (e.g., upstream regulators, NF-\(\kappa\)B subunits, and NF-\(\kappa\)B-responsive genes), and sAPP\(\alpha\) production (e.g., secretases) as potential targets for therapeutic intervention in AD.

In addition to perturbed calcium homeostasis, accumulating data strongly suggest major contributions of oxidative stress and mitochondrial dysfunction to the pathogenesis of neuronal degeneration in AD (for a review, see Refs. 71–73). Aberrant processing of APP may promote neurodegeneration by increasing levels of neurototoxic forms of A\(\beta\), which induce oxidative stress and disrupt calcium homeostasis, and/or by decreasing production of sAPP\(\alpha\), which exhibits protective activity (for a review, see Ref. 31). Our data suggest that, by enhancing levels of oxidative stress and promoting mitochondrial dysfunction, PS-1 mutations may sensitize neurons to A\(\beta\)-induced apoptosis. Levels of rhodamine 123 fluorescence and MTT reduction (measures of mitochondrial transmembrane potential and energy charge/redox state, respectively) were significantly decreased in PC12 cell lines expressing mutant PS-1 following exposure to A\(\beta\) compared with vector-transfected lines and lines overexpressing wild-type PS-1. In addition, levels of DC fluorescence (a measure of cellular peroxide levels) were significantly increased in cells exposed to A\(\beta\), an effect exacerbated by mutant PS-1. Activation of NF-\(\kappa\)B appears to play an important role in suppressing oxyradical production and preserving mitochondrial function in cells exposed to A\(\beta\), because treatment of cells with NF-\(\kappa\)B decoy DNA enhanced the A\(\beta\)-induced oxyradical accumulation and mitochondrial dysfunction and completely blocked the protective effect of sAPP\(\alpha\). Oxyradical stress and mitochondrial dysfunction in our paradigm may result from perturbed cellular calcium homeostasis, because elevation of [Ca\(^{2+}\)]\(\text{cyt}\) induced by A\(\beta\) was correlated with increased oxyradical levels and mitochondrial impairment and because agents that block calcium release from ER (dantrolene) or influx through voltage-dependent calcium channels (nifedipine) suppressed oxyradical accumulation and mitochondrial dysfunction and completely blocked the protective effect of sAPP\(\alpha\). Oxyradical stress and mitochondrial dysfunction in our paradigm may result from perturbed cellular calcium homeostasis, because elevation of [Ca\(^{2+}\)]\(\text{cyt}\) induced by A\(\beta\) was correlated with increased oxyradical levels and mitochondrial impairment and because agents that block calcium release from ER (dantrolene) or influx through voltage-dependent calcium channels (nifedipine) suppressed oxyradical accumulation and mitochondrial dysfunction (28). Moreover, sAPP\(\alpha\)s largely prevented A\(\beta\)-induced elevations of [Ca\(^{2+}\)]\(\text{cyt}\), and peroxide levels and stabilized mitochondrial transmembrane potential in cells expressing mutant PS-1; sAPP\(\alpha\) also protected the cells against thapsigargin-induced apoptosis. Collectively, the data suggest that sAPP\(\alpha\)s prevent apoptosis by stabilizing calcium homeostasis, suppressing oxyradical production, and preserving mitochondrial function.

Additional mechanisms whereby presenilin mutations may promote apoptosis are suggested by recent studies. For example, Kim and co-workers (74) reported that PS-1 and PS-2 are cleaved by a caspase-3 family protease in a neuroglial cell line induced to undergo apoptosis. Apoptosis induced by A\(\beta\) in neurons is blocked by inhibitors of caspases, a family of proteins believed to play important roles in the effector phase of apoptosis (64, 75). Caspase inhibitors also block apoptosis in PC12 cells expressing mutant PS-1. However, it is not known whether cleavage of PSs by caspases is mechanistically involved in apoptosis or is a consequence of the apoptotic process. Interactions of presenilins with APP have been reported (76, 77) and might influence indirect roles in promoting neuronal apoptosis. Enzymatic processing of APP is altered in AD such that levels of A\(\beta\) production (particularly the A\(\beta\)1-42 form) are increased and levels of sAPP\(\alpha\) are decreased. Levels of A\(\beta\)1-42 production are increased in cultured cells transfected with mutant presenilins, in transgenic mice expressing mutant PS-1, and in cultured fibroblasts from carriers of PS mutations (24, 78). It is unclear how presenilin mutations alter APP processing, but one possibility is that the altered processing is secondary to increased levels of cellular stress. In support of this hypothesis, we have shown that PS-1 mutations destabilize calcium homeostasis and enhance oxidative and metabolic stress in PC12 cells (Refs. 27 and 28 and this study), and other laboratories have shown that sustained elevations of [Ca\(^{2+}\)]\(\text{cyt}\) (79) and metabolic/oxidative stress (80) shift APP processing in favor of increased A\(\beta\) production. Prior studies have shown that enzymatic processing of APP is altered in AD such that there is reduced cleavage at the \(\alpha\)-secretase site and increased cleavage at the \(\beta\)- and \(\gamma\)-secretase sites (for a review, see Refs. 1 and 31). It is therefore likely that the increased A\(\beta\) production in cells expressing presenilin mutations is accompanied by reduced levels of sAPP\(\alpha\), and, indeed, there is evidence that sAPP\(\alpha\) levels are decreased in the cerebrospinal fluid of AD patients (81, 82), although data on sAPP\(\alpha\) levels in carriers of presenilin mutations have not yet been reported. Because sAPP\(\alpha\) is approximately 100-fold more potent than sAPP\(\beta\) in protecting cultured neurons against excitotoxic and oxidative insults (41), it is reasonable to consider that decreased levels of sAPP\(\alpha\) contribute to the proapoptotic actions of mutant PS-1. Consistent with this possibility, our data show that sAPP\(\alpha\) is very effective in protecting PC12 cells expressing mutant PS-1 against apoptosis induced by A\(\beta\).

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