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Overexpression of Amyloid- β Protein Precursor Induces Mitochondrial Oxidative Stress and Activates the Intrinsic Apoptotic Cascade

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Abstract

Alzheimer's disease (AD) is a debilitating cognitive disorder which is characterized pathologically by amyloid- β plaques and neurofibrillary tangles. Aberrant processing of amyloid beta protein precursor (A β PP) into amyloid- β fragments underlies the formation of senile plaques. Moreover, amyloid- β fragments, particularly A β_{42} , exert direct toxic effects within neurons including the induction of mitochondrial oxidative stress (MOS). Interestingly, individuals with Down Syndrome (DS) frequently develop early onset AD as a major co-morbid phenotype. One hypothesis for AD associated with DS involves the overexpression of wild type (WT) A β PP protein, due to its location on chromosome 21. However, the mechanism by which the overexpression of WT A β PP might trigger MOS and induce cell death is presently unclear. Here we show that transient overexpression of DsRed2-tagged A β PP (WT) in CHO cells induces the activation of caspase-3 and nuclear fragmentation indicative of apoptosis. A β PP localizes to the mitochondrial fraction of transfected CHO cells and its overexpression causes glutathione (GSH)-sensitive opening of the mitochondrial permeability transition pore (mPTP) and cytochrome c release. MOS and intrinsic apoptosis induced by A β PP were significantly inhibited by the co-expression of Bcl-2 or treatment with either GSH or Boc, a pan-caspase inhibitor. Furthermore, the mPTP inhibitor, cyclosporin A, also significantly protected CHO cells from apoptosis induced by A β PP overexpression. Finally, co-treatment with a β -secretase inhibitor did not significantly protect CHO cells from A β PP overexpression and A β_{42} levels were undetectable in transfected CHO cells. Therefore, the mechanism of A β PP induced MOS and apoptosis is independent of the production of A β_{42} . However, a γ -secretase inhibitor showed significant protection of the CHO cells against A β PP overexpression. Thus, suggesting a possible role of the A β PP intracellular domain in this apoptotic pathway. These data indicate that overexpression of WT A β PP is sufficient to induce MOS and intrinsic apoptosis, suggesting a novel pro-oxidant role for A β PP at the mitochondria in AD and DS disease pathologies.

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Keywords

Amyloid beta protein precursor (A β PP); Alzheimer's disease (AD); Down syndrome (DS); Glutathione (GSH); Oxidative stress; Apoptosis

Introduction

Alzheimer's disease (AD) is the most common form of dementia, currently affecting approximately five million people in the United States. This number is sure to increase as the elderly population continues to grow. The clinical symptoms of AD result from degeneration of cholinergic neurons in the cortex and hippocampus. AD pathology involves the aggregation of amyloid- β fibrils into amyloid plaques and the formation of neurofibrillary tangles. Amyloid or senile plaques are caused by the aberrant processing of amyloid beta protein precursor (A β PP) [1]. Neurofibrillary tangles are formed by aggregation of hyperphosphorylated tau protein [2]. In addition, amyloid- β fragments, particularly A β ₄₂, exert direct toxic effects within cells. These include Ca²⁺ dysregulation, mitochondrial dysfunction, and induction of oxidative stress, to name a few [3, 4, 5].

Down Syndrome (DS) occurs in approximately one in every 735 births in the United States [6]. DS individuals often display an early onset, AD-like dementia as a prominent co-morbid phenotype [7, 8]. DS is caused by a partial or full triplication of chromosome 21 (HSA21), the smallest of the human chromosomes, on which the A β PP gene is located [9, 10]. Triplication of HSA21 appears to cause many genes on that chromosome to be overexpressed by ~50% in multiple tissues, an effect which is recapitulated in the Ts65Dn mouse model of DS [11]. Currently it is not known how this increase in transcript levels results in the common features of DS. In regards to the relationship between the overexpression of specific genes on HSA21 and the pathology of DS, there are two leading hypotheses. The first is the gene dosage hypothesis, which states that due to the presence of an extra copy of HSA21 there is a corresponding 50% increase in the expression of genes on this chromosome that directly or indirectly alters the timing, pattern or extent of neuronal development [12]. The second hypothesis states that it is the expression of extra genes, regardless of their specific identity or function, that leads to disruption of genetic homeostasis, decreased buffering against environmental insults and ultimately, phenotypic alterations characteristic of DS [13]. Individuals with DS have been shown to have increased expression of brain A β PP and increased levels of amyloid- β in CSF, consistent with their predilection towards development of AD [14, 15].

Apoptotic cell death, mitochondrial dysfunction, and mitochondrial oxidative stress (MOS) contribute to both DS and AD pathologies [1, 9, 16, 17]. A β ₄₂ peptide is cytotoxic to neurons due to its ability to trigger cells to generate reactive oxygen species (ROS) [18]. Furthermore, amyloid- β has been shown to accumulate at mitochondria, leading to increased MOS and decreased mitochondrial metabolism [19]. In addition, wild type (WT) A β PP and swedish mutant A β PP have been shown to translocate to the mitochondria when overexpressed in a human cortical neuronal cell line [20]. Finally, in transgenic AD mice, the swedish mutant A β PP was shown to be localized to brain mitochondrial fractions [20].

The studies referenced above illustrate two major points. First, AD pathology either alone or on a background of DS, is intimately linked to MOS, mitochondrial dysfunction, and apoptosis. Second, A β ₄₂ peptide and WT or mutant forms of A β PP appear to accumulate at mitochondria and each of them has the potential to cause localized oxidative stress at this organelle. A major question that arises is what is the specific role of WT A β PP overexpression in the AD-like pathology observed in DS? Here, we show that overexpression of WT A β PP in CHO cells results in its localization to mitochondria and induction of MOS and intrinsic apoptosis. These data are consistent with the overexpression of WT A β PP playing a potentially toxic role at mitochondria. This novel pro-oxidant role of A β PP at mitochondria may be a significant factor in the AD pathology associated with DS.

Materials and Methods

Reagents and Antibodies

4,6-diamidino-2-phenylindole (DAPI), Hoechst dye (33258), kanamycin sulfate, ampicillin sodium salt, Ham's F12 nutrient mixture, staurosporine, LB broth and LB agar were purchased from Sigma Aldrich (St. Louis, MO). The β -secretase inhibitor IV was purchased from Millipore (Billerica, MA). The γ -secretase inhibitor was from EMD (Darmstadt, Germany). Glutathione monoethyl ester, Bax-6A7 monoclonal antibody, and cyclosporin A were from Calbiochem (San Diego, CA). Boc-D (OMe)-FMK was purchased from Alexis Biochemicals (Plymouth Meeting, PA). Anti-active caspase 3 and JM109 E. coli cells were from Promega (Madison, WI). Lipofectamine 2000, Opti-MEM media, DsRed2 plasmid, A β ₄₂ ELISA Kit, and Image-iT live mitochondrial transition pore assay kit were from Invitrogen (Carlsbad, CA). HRP-conjugated anti-mouse and anti-rabbit secondary antibodies, PVDF membranes, and ECL chemiluminescence detection reagents were from Amersham Biosciences (Piscataway, NJ). FITC and Cy3 conjugated anti-mouse and anti-rabbit secondary antibodies were from Jackson Immuno Research (Westgrove, PA). Anti-cytochrome c was from Biolegend (San Diego, CA). Anti-A β PP was from Abcam (Cambridge, MA). Anti-COX-IV was from Cell Signaling (Boston, MA). Anti-OPA-1 was purchased from BD (Franklin Lakes, NJ). Mitochondrial/Cytosolic cell fractionation kit was from Biovision (Mountain View, CA). BCA protein assay was from Pierce Chemical Co (Rockford, IL). DsRed2-WT A β PP plasmid was a generous gift from Dr. Xiongwei Zhu from Case Western Reserve University (Cleveland, OH).

Plasmid Preparation

DsRed2 and DsRed2-WT A β PP were transformed using 50ng of plasmid in JM109 *Escherichia coli* (E. coli) cells, and grown on LB agar plates containing 35 μ g/mL kanamycin sulfate at 37°C overnight. Starter cultures were grown in LB broth with 35 μ g/mL kanamycin sulfate for 8 h at 37°C, and diluted 1:250 into overnight cultures, for plasmid purification using the Qiagen Maxi Prep Kit (Valencia, CA). DNA concentrations were determined using a ThermoScientific NanoDrop 2000. Bcl-2 plasmid preparation was as listed above; however 50 μ g/mL of ampicillin sodium salt was added to LB agar and LB broth.

Cell Culture

K1-CHO (Chinese Hamster Ovary) cells were plated on 35-mm diameter plastic dishes in Ham's F12 media containing 10% fetal bovine serum, 2 mM L-glutamine and (100 U/mL/100 µg/mL) penicillin/streptomycin. Cells were cultured overnight at 37°C in 10% CO₂. The following day cells were prepared for transfection or treatment, at which point cultures were 60-80% confluent.

Transfection

DsRed2 or DsRed-WT AβPP were used at a concentration of 5 µg/mL. Bcl-2 plasmid was used at 10 µg/mL. Plasmids were transfected using a standard Lipofectamine 2000 protocol. CHO cells cultures were incubated with the plasmid/Lipofectamine 2000 mixture in Opti-MEM, for 6h at 37°C and 10% CO₂. Transfection was removed and cell cultures were placed in 1 mL of Ham's F12 media +/- appropriate treatments and incubated overnight at 37°C and 10% CO₂. All experiments were analyzed at 24 h post-transfection.

Immunocytochemistry and Fluorescence Imaging

CHO cells were washed once in 1X PBS (phosphate-buffered saline, pH 7.4) and then fixed with 1ml of 4% paraformaldehyde for 45 min at RT. Cells were then permeabilized and blocked with 5% BSA in 0.2% Triton X-100/1X PBS at RT for 1 h. Primary antibodies were diluted to working concentrations in 2% BSA in 0.2% Triton X-100/1X PBS and incubated overnight at 4°C. The following day cells were washed 5× with 1X PBS and cells were incubated for 1h at RT in secondary antibody at a dilution of 1:250 in 2% BSA in 0.2% Triton X-100/1X-PBS, DAPI (1:500) was also added to the secondary antibody solution to stain the nuclei. Next, cells were washed 5× in 1X PBS, and placed in anti-quench solution prepared from 1X PBS and 1-5 mg of p-phenylenediamine. Fluorescent images were captured using a 40X objective on a Zeiss Axiozoom microscope.

Mitochondrial/Cytosolic Subcellular Fractionation

Subcellular fractionation was performed as essentially described in the Mitochondrial/Cytosolic Kit protocol from BioVision.

Immunoblotting

Protein concentrations were determined using a BCA protein assay kit and absorbance was read using a Biotech Spectrophotometer. Sixty µg of protein were subjected to SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. Immunoblotting was performed by blocking PVDF membranes in 1X PBS; pH 7.4 with 0.1% Tween-20 (PBS-T) containing 1% BSA and 0.1% sodium azide for 1 h at RT. The primary antibody was diluted in blocking solution according to manufacturer's recommendations and incubated with membranes for 1 h. Membranes were then washed 5× over 30 min with PBS-T. Next, membranes were incubated for 1 h with appropriate HRP-conjugated secondary antibodies diluted in PBS-T, after which membranes were washed 5× over 30 min with PBS-T. This was followed by detection of immunoreactive proteins with enhanced chemiluminescence (ECL).

Mitochondrial PTP Assay

At 24 h post-transfection, CHO cells were washed once with Hank's balanced buffered salt solution (HBSS; pH 7.4) and cells were placed in 1 mL of Hank's buffer. Calcein AM (1mM), Hoechst 33342 (1mM), and cobalt (II) chloride hexahydrate (1 M) were added at 1 μ l each per well of cells. Cells were incubated at 37°C, 10% CO₂ for 15 min. Next, cells were washed once with HBSS and imaged live under fluorescence microscopy.

A β ₄₂ ELISA

CHO cells were transfected as described above and 24 h post-transfection cells were either subfractionated into mitochondrial fractions (described above) or whole cell lysates were obtained. In addition, media from transfected CHO cells was obtained for this assay. The ELISA was completed as per the manufacturer's protocol.

Statistical Analysis and Quantification of Data

Recorded values of apoptosis, caspase-3 activation, DNA fragmentation and mitochondria permeability transition pore (mPTP) opening were quantified from 30 images per treatment. Data is represented as mean $\pm$ SEM for the number (n) of independent experiments performed. Only transfected cells (DsRed2 fluorescent) were counted in each field and values are shown as a percentage of total transfected cells. Most experiments are representative of n=3. Statistical analysis was performed using one-way analysis of variance (ANOVA) and a post hoc Tukey's test. Differences were considered significant with a p value <0.05.

Results

Overexpression of A β PP at mitochondria induces caspase-3 activation and apoptosis

To determine the effects of overexpression of WT A β PP on cell viability, CHO cells were transfected with either DsRed2-WT A β PP or empty DsRed2 vectors. Overall transfection efficiency, assessed by DsRed2 fluorescence, was typically 30-50%. CHO cells transfected with DsRed2 alone displayed little-to-no nuclear fragmentation although some nuclei appeared relatively smaller, as shown in Figure 1A (left panels). However, in cells overexpressing WT A β PP (identified by DsRed2 fluorescence), many nuclei appeared markedly fragmented and condensed, indicative of apoptosis (Figure 1A, right panels, arrowheads). Furthermore, CHO cells which were not transfected with WT A β PP in the same field of Figure 1A generally did not display fragmented nuclei. To verify A β PP expression in transfected cells, an immunoblot was performed on whole cell lysates from transfected CHO cells and tubulin was utilized as a loading control. Full length A β PP appeared as a doublet and although there appeared to be a small amount of endogenous CHO cell A β PP detected in DsRed transfected cells, there was a substantial increase in A β PP expression in cells transfected with DsRed-tagged A β PP (Figure 1B). In addition, transfected CHO cells were fractionated and immunoblotted for full length A β PP which localized extensively to the mitochondria (Figure 1C). Interestingly, an immunoblot analysis of the mitochondrial fusion GTPase, OPA-1, as a marker for mitochondrial fractions, actually revealed decreased protein expression in A β PP transfected cells (Figure 1C). This

finding is in agreement with a previous study showing that A β PP overexpression induces a decrease in OPA-1 protein levels in human neuroblastoma cells [21]. In order to provide further evidence of apoptotic cell death due to overexpression of A β PP, we stained the transfected CHO cells for active caspase-3, an executioner caspase of the apoptotic cascade. Overexpression of WT A β PP induced significant activation of caspase-3 compared to DsRed2 overexpression (Figure 2, arrowheads). In addition, cells transfected with WT A β PP and staining positive for active caspase-3 (i.e., these cells appear yellow) displayed condensed and fragmented nuclei. Quantification of these experiments demonstrated a significant increase in nuclear fragmentation and caspase-3 activation in WT A β PP overexpressing cells versus DsRed2 control cells (see Figures 6A and 6B).

A β PP-induced apoptosis is attenuated by caspase inhibition, Bcl-2 co-expression, or supplementation with GSH

To establish if the apoptosis induced by WT A β PP was caspase-dependent, we next examined the protective effects of Boc, a pan-caspase inhibitor. CHO cells overexpressing WT A β PP displayed an increase in active caspase-3 and nuclear fragmentation and condensation (Figure 3, left panels, arrowheads). However, CHO cells treated with Boc, in addition to transfection with WT A β PP, displayed significantly fewer cells with immunoreactivity for active caspase-3 or showing apoptotic nuclei compared to overexpression of A β PP alone (Figure 3, right panels). This protective effect of the caspase inhibitor was highly statistically significant as shown in Figures 6A and 6B.

Intrinsic apoptosis involves the inhibition of pro-survival Bcl-2 family proteins. These pro-survival proteins include Bcl-2, Bcl-X_L, Bcl-w, A1 and Mcl-1. It has recently been shown that A β PP can associate with and potentially inhibit Bcl-2 directly through a heat shock protein complex-dependent interaction [22]. Therefore, we examined the effects of co-expression of Bcl-2 on the ability of WT A β PP to induce apoptosis. CHO cells overexpressing WT A β PP showed pronounced caspase-3 activation as described above (Figure 4A, left panels, arrowheads). In contrast, CHO cells co-transfected with Bcl-2 and WT A β PP displayed significantly less caspase-3 activation and apoptosis when compared to cells overexpressing A β PP alone (Figure 4A, right panels). To maximize the probability that co-transfected CHO cells were expressing both WT A β PP and Bcl-2, the amount of Bcl-2 plasmid added to transfection reactions was twice that of the WT A β PP plasmid. Furthermore, Figure 4B shows an immunoblot demonstrating Bcl-2 expression and localization to mitochondria in co-transfected CHO cells. The protective effects of Bcl-2 co-expression against A β PP-induced apoptosis are quantified in Figures 6A and 6B.

It has been previously shown that Bcl-2 displays an antioxidant-like function at mitochondria [23], which could be an additional mechanism of protection from A β PP overexpression beyond merely inhibiting intrinsic apoptosis. Therefore, we hypothesized that the overexpression of WT A β PP was inducing ROS, and to test this we examined the protective effects of GSH, a key endogenous cellular antioxidant. CHO cells overexpressing WT A β PP showed the typical increase in apoptosis, indicated by induction of active caspase-3 and nuclear fragmentation/condensation (Figure 5, left panels, arrowheads). However, CHO cells treated with GSH in addition to transfection with WT A β PP showed

significantly less apoptosis (Figure 5, right panels). These data are quantified in Figures 6A and 6B, where GSH significantly decreased active caspase-3 and nuclear fragmentation induced by WT A β PP. These results suggest that overexpression of WT A β PP induces oxidative stress at the level of the mitochondria, which subsequently leads to apoptosis.

A β PP causes Bax-independent cytochrome c release from mitochondria

The release of cytochrome c from mitochondria is one of the initiating steps involved in the intrinsic apoptotic pathway. To confirm that the mechanism of cell death induced by A β PP involved the intrinsic apoptotic pathway, we immunoblotted for cytochrome c in mitochondrial and cytosolic fractions of transfected CHO cells. Cells transfected with empty DsRed2 displayed cytochrome c localization exclusively within mitochondrial fractions (Figure 7). However, overexpression of WT A β PP induced cytochrome c release into the cytosol, indicated by the appearance of cytochrome c in the cytosolic fraction. The relatively small magnitude of cytochrome c release into the cytosol may be due to the transfection efficiency, since only those cells transfected with A β PP would be actively releasing cytochrome c. CHO cells treated with GSH in addition to transfection with A β PP showed diminished cytochrome c release (Figure 7). These results are consistent with cytochrome c release occurring as a result of A β PP induction of MOS.

The release of cytochrome c often occurs via a Bax-dependent pore formed in the outer membrane of mitochondria [24]. To determine if Bax is involved in the apoptotic pathway induced by overexpression of WT A β PP, transfected CHO cells were stained with a conformation specific antibody against active Bax (6A7). As a positive control, cells were treated with staurosporine, an inducer of apoptosis known to activate Bax. CHO cells overexpressing WT A β PP failed to show any discernible activation of Bax (data not shown), however treatment with staurosporine caused a marked increase in active Bax as expected (data not shown). Therefore, the release of cytochrome c and subsequent activation of the apoptosis pathway induced by the overexpression of WT A β PP occurs via a GSH-sensitive but Bax-independent mechanism.

A β PP induces a Bcl-2- and GSH-sensitive opening of the mPTP

We have shown that the overexpression of WT A β PP and its localization to mitochondria induces intrinsic apoptosis of CHO cells which is significantly attenuated by either Bcl-2 co-expression or addition of exogenous GSH. These findings suggest that WT A β PP overexpression induces MOS. When levels of ROS are increased, especially within mitochondria, the mitochondrial permeability transition pore (mPTP) becomes highly susceptible to calcium-induced opening [25]. Moreover, the mPTP is a prominent Bax-independent mechanism for cytochrome c release [25]. Thus, ROS-induced opening of the mPTP can be used as an indicator of MOS. To determine if A β PP induced opening of the mPTP, transfected CHO cells were utilized in a cobalt/calcein assay to measure mPTP induction. In this assay, if the mPTP is activated, then CoCl₂ is released from the matrix of mitochondria into the cytosol allowing it to react with and quench the fluorescence (green) signal of calcein which is localized in the cytoplasm. CHO cells transfected with empty DsRed2 maintained high levels of calcein staining in the cytoplasm, whereas cells transfected with A β PP displayed diminished levels of calcein fluorescence (Figure 8A,

upper panels). These data indicate that overexpression of WT A β PP is capable of activating the mPTP. As a positive control, CHO cells transfected with DsRed2 were treated with ionomycin, a calcium ionophore which increases intracellular Ca²⁺, thus triggering the activation of the mPTP. Quantification of the percentage of transfected cells displaying mPTP opening (i.e., loss of calcein fluorescence) is shown in Figure 8B. CHO cells transfected with DsRed2 and treated with ionomycin showed a dramatic increase in opening of the mPTP compared to DsRed2 transfection alone. Overexpression of A β PP induced a similar percentage of cells with activated mPTP as treatment with ionomycin. Upon supplementation of A β PP overexpressing cells with GSH or co-transfection with Bcl-2, the mPTP activation was blocked (Figure 8A, lower right panel and Figure 8B). These findings are consistent with GSH inhibiting A β PP-induced cytochrome c release (see Figure 7). In contrast, A β PP overexpressing cells treated with Boc did not show any reduction in mPTP opening (Figure 8A, lower left panel and Figure 8B), indicating that the inhibitory action of Boc on caspases occurs downstream of mPTP opening. Collectively, these results demonstrate that A β PP triggers a GSH-sensitive opening of the mPTP (i.e., MOS) that likely contributes to cytochrome c release upstream of caspase activation.

The mPTP inhibitor, cyclosporin A, protects cells from A β PP-induced apoptosis

To determine if opening of the mPTP is required for A β PP-induced apoptosis, we tested whether cyclosporin A, an mPTP inhibitor, would protect transfected CHO cells. CHO cells overexpressing WT A β PP and treated with either 1 μ M cyclosporin A or 10 μ M cyclosporin A displayed a significant decrease in immunoreactivity for active caspase-3 compared to cells overexpressing A β PP alone (Figures 9A and 9B). Thus, mPTP opening appears to be a key mechanism underlying A β PP-induced apoptosis.

Overexpression of A β PP induces apoptosis independent of A β ₄₂

In order to verify that the effects of A β PP overexpression were not due to the accumulation of A β ₄₂ we completed an ELISA on CHO cells transfected for 24 h with either DsRed or A β PP. The levels of A β ₄₂ were undetectable in whole cell lysates, mitochondrial fractions, and media from the transfected CHO cells (data not shown). In addition, transfected CHO cells were treated with a β -secretase inhibitor to determine if the inhibition of A β ₄₂ production would protect these cells from apoptosis induced by A β PP overexpression. In accordance with the ELISA data, the β -secretase inhibitor did not provide any discernible protection from A β PP overexpression (Figure 10). Furthermore, we tested a γ -secretase inhibitor to determine if the A β PP intracellular domain (AICD) might play a role in the apoptotic cascade induced by A β PP overexpression in CHO cells. Interestingly, the γ -secretase inhibitor provided statistically significant protection from apoptosis induced by the overexpression of A β PP (Figure 10).

Discussion

The underlying pathology of AD has been linked to the aberrant processing of A β PP into soluble amyloid- β fibrils, and the gene for A β PP is found on chromosome 21, suggesting a role for A β PP in DS etiology. Interestingly, individuals with DS frequently display early onset AD and have increased A β serum levels [14, 15]. Furthermore, in the Ts65Dn mouse

model of DS, which is trisomic for A β PP, a reduction of A β serum levels has been correlated to an improvement in learning and memory [26]. In addition, genetic reduction of A β PP from 3 copies to 2 copies improves endosomal pathology observed in the Ts65Dn mouse [27]. Furthermore, treatment of the Ts65Dn mouse with Vitamin E has been correlated to an improvement in learning and memory, suggesting that oxidative stress plays a significant role in the pathology of this DS model [28]. Although A β PP is the precursor for amyloid- β it has been hypothesized that the downstream effects of this full length protein induce many of the cognitive hallmarks of AD and AD in DS. The observations made in this study illustrate that overexpression of A β PP and its localization to mitochondria induces MOS in the form of GSH-sensitive mPTP opening. The mitochondrial permeability transition activates a Bax-independent release of cytochrome c, triggering the intrinsic apoptotic cascade (Figure 10). The toxic effects of A β PP are significantly attenuated by compounds that target the intrinsic apoptotic pathway and oxidative stress, such as Bcl-2 (pro-survival), Boc (pan-caspase inhibitor), cyclosporin A (mPTP inhibitor), and GSH (antioxidant). Given that GSH and Bcl-2 prevent mPTP opening induced by A β PP but Boc does not, we can definitively place GSH-sensitive mPTP opening (i.e., MOS) upstream of caspase activation. Thus, opening of the mPTP appears to be the key event in triggering A β PP-induced apoptosis. Furthermore, we demonstrate that this apoptotic pathway is independent of the A β ₄₂ fragment but cannot exclude the involvement of the AICD fragment.

Our data are consistent with a number of previous studies. For example, one study using SH-SY5Y neuroblastoma cells showed that the overexpression of WT A β PP caused the cells to become more susceptible to oxidative stress [29]. In addition, studies have also shown that the overexpression of A β PP can induce apoptosis, increase intracellular Ca²⁺ levels, and trigger axonal pruning [30]. Finally, Bcl-2 has been shown to be protective against A β -induced toxicity in Gt1-7 neural cells [31].

Recent studies have provided evidence for a critical role of mitochondrial GSH in preventing accumulation of ROS and subsequent induction of apoptosis [32]. Other studies have shown that A β PP is targeted to mitochondria where it can induce MOS and disrupt mitochondrial function; however, the direct mechanism has remained elusive [20, 21, 29]. We examined the effects of A β PP on mitochondrial GSH and although a trend was evident for the overexpression of WT A β PP to deplete mitochondrial GSH, our results were highly variable and therefore never reached significance (data not shown). These results could be due to the CHO cell transfection efficiency being only 30-50%. For example, if A β PP induced a 50% reduction of mitochondrial GSH in transfected cells which made up 50% of the culture, this would result in only a 25% overall reduction in mitochondrial GSH detected in the entire culture. Therefore, our assay system may not be sensitive enough to detect this effect in the transfected CHO cells. However, the localization of A β PP to mitochondria and depletion of mitochondrial GSH provide a plausible mechanism of A β PP-induced MOS. Future studies are necessary to determine the putative mechanism by which A β PP leads to a depletion of mitochondrial GSH. In this context, we have shown that Bcl-2 is a GSH-binding protein and a regulator of the mitochondrial GSH pool [33]. Moreover, Bcl-2 can interact with A β PP in a complex with heat shock proteins [22]. These previous studies provide some insight into the potential mechanism by which A β PP may induce

mitochondrial GSH depletion. In particular, it will be interesting to determine if A β PP disrupts the ability of Bcl-2 to bind GSH and to regulate the mitochondrial GSH pool.

We provide further evidence of a critical role for MOS caused by the overexpression of WT A β PP by showing that it induces the GSH-sensitive opening of the mPTP. Previous studies have demonstrated that ROS can oxidize the adenine nucleotide translocase (ANT), causing it to bind cyclophilin D and triggering opening of the mPTP. Opening of the mPTP can lead to outer mitochondrial membrane rupture and the release of cytochrome c and downstream activation of caspases [34]. Through the use of cyclosporin A, we further provide evidence for the involvement of the mPTP in the apoptotic cascade induced by A β PP. Cyclosporin A inhibits cyclophilin D by preventing its peptidyl-prolyl cis-trans isomerase activity, which is required for it to function as a component of the mPTP [34]. Our data indicate that CHO cells overexpressing WT A β PP are significantly protected by cyclosporin A. In addition, the activation of the mPTP is prevented by Bcl-2 and GSH, but not Boc, again supporting the central role of MOS in this apoptotic pathway.

Consistent with previous studies our data indicate an A β ₄₂-independent mechanism of MOS and apoptosis induced by the overexpression of WT A β PP since we could not detect A β ₄₂ in mitochondrial fractions of transfected CHO cells and a β -secretase inhibitor failed to protect cells from apoptosis. In a previous study using mice which overexpress WT A β PP, hippocampal degeneration was similarly shown to be independent of A β ₄₂ levels [35]. However, this study did find an increase in the AICD, although the specific role of this fragment in mediating the hippocampal degeneration was not investigated [35]. Interestingly, γ -secretase has been shown to process A β PP locally within mitochondria into the AICD fragment, thus providing a source of this potentially pathogenic A β PP fragment within these organelles [36]. Our data indicate that inhibition of γ -secretase activity has a significant protective effect against apoptosis induced by overexpression of WT A β PP in CHO cells; however, additional studies are necessary to discern the role of the AICD in promoting MOS and apoptosis in this system. It is noteworthy that mice transgenic for the AICD display Alzheimer's disease-like pathology [37], yet a definitive role for the AICD in causing MOS and permeability transition has not been established.

In future work, we plan to investigate the role of MOS and intrinsic apoptosis in mice which overexpress WT A β PP and examine these pathways in the Ts65Dn mouse model of DS. In addition, elucidating the direct mechanism by which A β PP (or the AICD) induces mitochondrial GSH depletion will provide additional insight into the toxicity of this protein. The novel pro-oxidant role of A β PP at mitochondria elucidated here may play a key role in AD and AD associated with DS, providing a possible common pathway underlying the pathologies of these two diseases.

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Figure 1. Overexpression of WT AβPP induces apoptosis

(A) Top panel left; DsRed-2 (red) transfected CHO cells, nuclei (blue) DAPI. Arrows indicate healthy transfected cells. Top panel right; AβPP (red) transfected CHO cells, nuclei are stained with DAPI (blue), arrows indicate apoptotic cells. Bottom panels; decolorized DAPI. (B) Immunoblot for AβPP in transfected CHO cell lysates, tubulin loading control. AβPP molecular weight was approximately 160 kDa consistent with the full length protein containing a DsRed tag. (C) Immunoblot analysis for AβPP (~160 kDa) and OPA-1 in transfected CHO cell mitochondrial fractions.

Figure 2. Overexpression of WT A β PP induces the activation of caspase-3

Top panel; CHO cells transfected with either DsRed2 (red, left) or A β PP (red, right). Arrows indicate cells transfected with A β PP, which are also positive for active caspase-3 (shown in the bottom panel). Bottom panels; CHO cells transfected with either DsRed2 (red; left) or A β PP (red; right), stained for active caspase-3 (green), nuclei (blue) DAPI. Arrows indicated cells stained positive for active caspase-3 and transfected with A β PP.

Figure 3. Boc protects from apoptosis induced by the overexpression of WT AβPP

Top panel left; CHO cells transfected with AβPP (red), stained for active caspase-3 (green), and nuclei (blue) DAPI. Arrows indicated cells stained positive for active caspase-3. Top panel right; CHO cells transfected with AβPP (red) treated with BOC (50 μM) stained for active caspase-3 (green), and nuclei (blue), DAPI. Bottom panel; decolorized DAPI, arrows indicated apoptotic nuclei which also stained positive for active caspase-3.

Figure 4. Bcl-2 protects from apoptosis induced by the overexpression of WT A β PP
 (A) Top panel left; A β PP (red) transfected CHO cells, and nuclei (blue) DAPI, arrows indicate A β PP transfected CHO cells also stained positive for active caspase-3 (shown in the bottom panel). Top panel right; A β PP (red) transfected CHO cells, co-transfected with Bcl-2, and nuclei (blue) DAPI. Bottom panel left; A β PP (red) transfected CHO cells, stained for active caspase-3 (green), and nuclei (blue) DAPI, arrows indicate cells transfected with A β PP and positive for active caspase-3. Bottom panel right; A β PP (red) transfected CHO cells, co-transfected with Bcl-2, stained for active caspase-3, and nuclei (blue) DAPI. (B) Immunoblot of CHO cell mitochondrial/cytosolic fractions for Bcl-2 indicating transfection, Cox-IV loading control.

Figure 5. GSH protects from apoptosis induced by the overexpression of WT AβPP

Top panel left; CHO cells transfected with AβPP (red), stained for active caspase 3 (green) and nuclei (blue) DAPI. Arrows indicate CHO cells transfected with AβPP stained positive for active caspase-3. Top panel right; CHO cells transfected with AβPP (red), treated with 2 mM GSH monoethyl ester, stained for active caspase-3 (green), and nuclei (blue) DAPI. Bottom panel; decolorized DAPI images, arrows indicate apoptotic nuclei which also stained positive for active caspase-3.

Figure 6. Quantification of nuclear fragmentation and active caspase-3

(A) Quantification of nuclear fragmentation in transfected CHO cells. * $p < 0.05$ versus AβPP, *** $p < 0.001$ versus AβPP, # $p < 0.001$ versus DsRed2. A total of 30 images per treatment were captured, $n = 3$. Each treatment is expressed as percentage of total cells, only transfected cells were quantified. (B) Quantification of active caspase-3 in transfected CHO cells. ** $p < 0.01$ versus AβPP, *** $p < 0.001$ versus AβPP, # $p < 0.001$ versus DsRed2. A total of 30 images per treatment were captured, $n = 3$. Each treatment is expressed as percentage of total cells, only transfected cells were quantified.

Figure 7. Overexpression of WT AβPP induces GSH-sensitive cytochrome c release
Immunoblot of mitochondrial/cytosolic fractions for cytochrome C in CHO cells transfected with DsRed2, AβPP, or AβPP with 2 mM GSH treatment. Cox-IV is shown as a loading control.

Figure 8. WT AβPP activates the mPTP; GSH and Bcl-2 prevent mPTP opening

(A) CHO cells transfected with DsRed2 (top, left), AβPP (top, right), AβPP with 50 μM Boc treatment (bottom, left) and AβPP with 2 mM GSH treatment (bottom, right). Transfected/treated cells were utilized in a cobalt/calcein assay to measure the activation of the mPTP. Mitotracker (red), calcein AM staining (green, yellow with merged mitotracker), the absence of calcein AM staining indicates the activation of the mPTP due to the release of CoCl₂ into the cytoplasm. (B) Quantification of mPTP activation. DsRed2 + Ionomycin was utilized as a positive control. ** p<0.01 versus AβPP, ***p<0.001 versus AβPP #p<0.001 versus DsRed2. A total of 30 images per treatment were captured, n=3. Data is represented as a percentage of total cells.

Figure 9. Cyclosporin A protects from apoptosis induced by the overexpression of WT AβPP (A) CHO cells transfected with DsRed2 (top, left), AβPP (top, right), AβPP treated with 1 μM CSA (bottom, left), and AβPP treated with 10 μM CSA (bottom, right). All images show transfection (red) active caspase 3 (green) and nuclei (blue) DAPI. (B) Quantification of apoptosis indicated by active caspase 3. ** p<0.01 versus AβPP, ***p<0.001 versus AβPP, #p<0.001 versus DsRed2. A total of 30 images per treatment were captured, n=3. Each treatment is expressed as percentage of total cells, only transfected cells were quantified.

Figure 10. Overexpression of AβPP induces apoptosis independent of Aβ₄₂ levels
CHO cells transfected with AβPP (Con), AβPP treated with 250 nM β-secretase inhibitor (β-Sec Inh), or AβPP treated with 500 nM γ-secretase inhibitor (γ-Sec Inh). Quantification of apoptosis indicated by active caspase 3, n=3. *p<.05 AβPP vs γ-Sec Inh, data were analyzed using a Student's T-test.

Figure 11. Model of apoptosis induced by the overexpression of WT AβPP

Our data support a mechanism by which overexpression of WT AβPP leads to increased ROS, mPTP activation, Bax-independent cytochrome C release, and caspase-dependent apoptosis.