Neurotoxic Mechanisms Caused by the Alzheimer’s Disease-linked Swedish Amyloid Precursor Protein Mutation

OXIDATIVE STRESS, CASPASES, AND THE JNK PATHWAY

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Autosomal dominant forms of familial Alzheimer’s disease (FAD) are caused by mutations of the amyloid precursor protein (APP) gene and by mutations of the genes encoding for presenilin 1 or presenilin 2. Simultaneously, evidence is provided that increased oxidative stress might play a crucial role in the rapid progression of the Swedish FAD. Here we investigated the effect of the Swedish double mutation (K670M/N671L) in the β-amyloid precursor protein on oxidative stress-induced cell death mechanisms in PC12 cells. Western blot analysis and cleavage studies of caspase substrates revealed an elevated activity of the executor caspase 3 after treatment with hydrogen peroxide in cells containing the Swedish APP mutation. This elevated activity is the result of the enhanced activation of both intrinsic and extrinsic apoptosis pathways, including activation of caspase 2 and caspase 8. Furthermore, we observed an enhanced activation of JNK pathway and an attenuation of apoptosis by SP600125, a JNK inhibitor, through protection of mitochondrial dysfunction and reduction of caspase 9 activity. Our findings provide evidence that the massive neurodegeneration in early age of FAD patients could be a result of an increased vulnerability of neurons through activation of different apoptotic pathways as a consequence of elevated levels of oxidative stress.

Alzheimer’s disease is the most frequent neurodegenerative disorder in the aged population and is characterized by the presence of senile plaques and neurofibrillary tangles in the brain. Senile plaques are deposits of amyloid-β-protein (Aβ), which arises by proteolysis from the amyloid precursor protein (APP) through an initial β-secretase cleavage by the aspartyl protase beta-site APP-cleaving enzyme (1, 2) followed by an intramembranous cut of γ-secretase, which resides in a high molecular weight complex composed of presenilin (3), nicastrin (4–6), PEN-2 (5), and probably Aph-1 (6).

Several mutations in the APP and presenilin genes cause some forms of familial Alzheimer’s disease (FAD) (7–9). These mutations alter APP processing with respect to an enhanced Aβ production (10, 11) and have been associated with an increased vulnerability to cell death (12–15). β-Secretase cleavage of the Swedish double mutation form of APP (APPsw; K670M/N671L) occurs in Golgi-derived secretory vesicles, whereas wild-type APP (APPwt) must be reinternalized before β-secretase cleavage (16). This altered APP production leads to a 3–6-fold increased Aβ production of both Aβ(1–40) and Aβ(1–42) (17, 18), which probably possess the same structural peptide architecture (19, 20). Recent reports have demonstrated an intraneuronal Aβ accumulation in transgenic mice expressing FAD proteins (21). Alzheimer mice transgenic for APPsw mutation, but not wild-type mice, exhibited an age-dependent increase in soluble Aβ(1–40) and Aβ(1–42) levels and progressive amyloid deposition in brain (22, 23).

Despite all of these findings, the underlying mechanisms responsible for the massive neurodegeneration in early age of FAD patients are still not completely understood. Persuasive evidence indicates that oxidative stress plays an important role in the neuropathological process in AD (24–27). AD-related mutations probably enhance oxidative stress. Increased oxidative stress levels have been found in the temporal inferior cortex from Swedish FAD patients (28), and brains of mice transgenic for human presenilin 1 show reduced antioxidative enzyme activity (29).

Moreover, convincing evidence indicates that Aβ is neurotoxic, probably via an apoptotic pathway (12, 30–34). Aβ is believed to play a major role in promoting neuronal degeneration and death by rendering neurons vulnerable to age-related increases in levels of oxidative stress and impairments in cellular energy metabolism (32, 35). New evidence indicates that reactive oxygen species-induced cellular events implicate the activation of mitogen-activated protein kinases (36, 37). Oxidative stress may cause the activation of c-Jun N-terminal kinase (JNK; also known as stress-activated protein kinase) in degenerating neurons in AD (38). An involvement of JNK pathway by the induction of Fas ligand in Aβ-induced neuronal apoptosis was described by Morishima et al. (39). Activation of JNK and p38 associated with amyloid deposition was observed in mice transgenic for the human APPsw mutation (40). The inhibition of the JNK pathway was proposed as a potential therapeutic target in AD (41).

Caspases can be divided into initiator caspases and effector caspases based on the presence of a large prodomain at their

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EXPERIMENTAL PROCEDURES

Materials—Phospho-JNK (Thr<sup>183</sup>/Tyr<sup>185</sup>) and phospho-c-Jun (Ser<sup>63</sup>) antibodies were purchased from Cell Signaling Technology, and cytochrome c antibody was from BD Biosciences. Rat-specific caspase 3 and caspase 9 antibodies were also purchased from Cell Signaling Technology. Caspase 2 antibody was purchased from Alexix. Caspase 8 antibody was from Biocat. The caspase subunits were purchased from Calbiochem (Ac-DEVD-p-nitroaniline (pNA), Ac-VEID-p-Na, Ac-VDVAD-pNA, and Ac-IETD-pNA for caspase 3, caspase 6, caspase 2, and caspase 8, respectively). JNK inhibitor II (SP600125, anthraxa (1.9-ED-pyrazol-6(2H)-one) was from Calbiochem.

Cell Culture and Transfection—The generation and characterization of PC12 cell lines expressing human APP<sup>wt</sup>, mutant APP (APP<sup>sw</sup>)—

Measurement of Caspase Activity—PC12 cell cultures were plated at a density of 5 × 10<sup>5</sup> cells/well (4 cm diameter) and treated with H<sub>2</sub>O<sub>2</sub>. Cells were harvested and lysed in SDS sample buffer: Tris-HCl (62.5 mM, pH 6.8), 2% SDS, 10% glycerol, 50 mM dithiothreitol, 0.01% bromphenol blue. After sonicating and boiling, proteins were separated by electrophoresis on a 10% polyacrylamide gel. The separated proteins were transferred to a polyvinylidene difluoride membrane (ImmobilonTM-P; Millipore). These membranes were stained with Ponceau S red (reversible stain) to visualize the proteins. Nonspecific sites on the membranes were blocked with 5% fat-free milk in Tris-buffered saline, 0.1% Tween 20 (TBST). The phosphorylated JNK was visualized with the appropriate primary phosphoantibody, phospho-stress-activated protein kinase/JNK (Thr<sup>183</sup>/Tyr<sup>185</sup>), which detects stress-activated protein kinase/JNK only dually phosphorylated at Thr<sup>183</sup>/Tyr<sup>185</sup>. The phosphorylated c-Jun was detected by a phospho-c-Jun (Ser<sup>63</sup>) antibody. After thorough washing with TBST, membranes were covered with ECL<sup>TM</sup> detection reagents and quickly exposed to an autoradiography film. Membranes were routinely stripped for actin control.

Caspase Immunoblot Analysis—PC12 cell cultures were plated at a density of 5 × 10<sup>5</sup> cells/well (10 cm diameter) and treated with H<sub>2</sub>O<sub>2</sub>. After the indicated periods of time, the medium was aspirated, and cells were processed for Western blotting with rat-specific cleaved caspase 3, caspase 8, and caspase 9 antibodies. Blots were scanned (Umax Astra 8). The caspase subunits were identified by densitometric analysis.

Determination of Cytochrome c Release—The amount of cytochrome c released from the mitochondrial intermembrane space into the cytosol was determined by dihydrotestosterone (DHT). Briefly, 5 × 10<sup>5</sup> cells were exposed to oxidative stress for different periods of time. After washing with ice-cold phosphate-buffered saline, cells were resuspended in permeabilization buffer containing 75 mM NaCl, 1 mM Na<sub>2</sub>HPO<sub>4</sub>, 8 mM NaPO<sub>4</sub>, 250 mM sucrose, 1 mM phenylmethylsulfonyl fluoride, additional protease inhibitors, and 0.05% digitonin. Following a centrifugation step at 800 × g at 4 °C for 10 min, the supernatant was removed from the pellet consisting of mitochondria and cellular debris. The supernatant containing cytoplasmic proteins was purified by centrifugation at 13,000 × g at 4 °C for 10 min. Equal amounts of protein (10 µg) were loaded on an 18% acrylamide gel and separated by SDS-PAGE. After immunoblotting with a monoclonal cytochrome c antibody, polyvinylidene difluoride membranes were stripped and reprobed either for COX4 or actin, ensuring equal protein loading and the absence of mitochondrial contamination.

Determination of Mitochondrial Membrane Potential—PC12 cell cultures were plated the day before at a density of 2 × 10<sup>5</sup> cells/well in a 24-well plate. The cells were pretreated for 1 h with the JNK inhibitor SP600125, and H<sub>2</sub>O<sub>2</sub> was added for 6 h. The mitochondrial membrane potential of the cell mitochondrial membrane was measured using the dye rhodamine 123. The dye was added to the cell culture medium at a concentration of 0.4 µM for 15 min. The cells were washed twice with Hanks’ balanced salt solution, and the fluorescence was determined with a fluorescence reader (Victor Multilabel counter; PerkinElmer Life Sciences). Transmembrane distribution of the dye depends on the mitochondrial membrane potential (ΔΨ<sub>m</sub>).

Statistical Analysis—Data are given as means ± S.E. For statistical comparison, paired t test, Student's t test, or one-way ANOVA followed by Tukey's post hoc test or two-way ANOVA were used. p values less than 0.05 were considered statistically significant.

RESULTS

Generation and Characterization of PC12 Cell Lines Overexpressing APP<sup>wt</sup> and APP<sup>sw</sup>—To investigate the effects of APP overexpression in oxidative stress-induced apoptosis, we used
APPwt-bearing cells (28.4 ± 4.7%) and vector control cells (21.3% ± 4.3%). Different clones with the same transfectant behave similarly in their sensitivity to H₂O₂-induced cell death (Fig. 1C).

**Oxidative Stress Induces Activation of Caspase 2 in PC12 Cells**—Oxidative stress in the human brain has been implicated as one major cause of neuronal cell loss in AD patients. However, the exact mechanism still remains unknown. Aβ appears to bind redox-active metals like zinc, copper, or iron with high affinity, resulting in production of hydrogen peroxide and autoxidation of the metallopeptide complex (52, 53). The signaling cascades activated following the oxidative stress have not been widely studied. Therefore, we studied the implication of caspase 2 by measuring the activity and by Western blotting. Cleavage of the photometric substrate Ac-VDVAD-p-nitroanilide by cytosolic protein extracts indicates the presence of caspase 2 protease activity in the cultures after exposure to hydrogen peroxide. The activity was already induced early in APPsw cells within 2 h of induction (Fig. 2A). Caspase 2 activity was continuously elevated over time compared with APPwt and vector-transfected cells. Maximum caspase 2 activity was measured after 2 h, with a 4-fold higher activity in APPsw cells than in APPwt and vector PC12 cells, respectively (Fig. 2B).

The mechanism of activation of caspasces occurs by a sequential cleavage of the zymogen to release the large and the small cleavage products. In accordance with our findings from the determination of activity, Western blotting confirmed cleavage of caspase 2 within 2 h of hydrogen peroxide induction (Fig. 2C). Again, the activation of caspase 2 was noticeably higher in APPsw cells compared with vector-transfected controls.

**Involvement of Caspase 8**—Apoptosis associated with the Fas/tumor necrosis factor receptor family of death receptors requires caspase 8 activity and adaptor proteins such as FADD. The involvement of caspase 8 in processing of APP during apoptosis by caspase 8 has been reported recently (46). The blocking of neuronal death by caspase 8 inhibitor IETD-fmk in Aβ-induced cell death was also demonstrated (54). Hence, we examined the activation of caspase 8 in our transfected cell lines overexpressing intracellular high levels of Aβ in order to ascertain the involvement of this initiator caspase in oxidative stress. We utilized Ac-IETD-pNA to measure caspase 8 activity in lysates of treated cells. In parallel to the early activation of caspase 2, caspase 8 activity increased about 3-fold after 2 h of stress induction in PC12 cells expressing the Swedish double mutation and was significantly enhanced in a time-dependent manner compared with vector-transfected controls (Fig. 3, A and B). Activation of caspase 8 involves a two-step proteolysis: the cleavage of the procaspase 8 to generate a 43-kDa fragment and a 12-kDa fragment that is further processed to 10 kDa. The large fragment is then cleaved to yield p26. Here, we used an antibody recognizing the 26-kDa large fragment of caspase 8. Western blot analysis showed a notable expression of active caspase 8 even in the untreated APPsw PC12 cells. Over the whole time period, the protein expression in APPsw cells was continuously higher compared with vector control (Fig. 3C).

In addition, we analyzed the activities of caspase 1 and caspase 6 using AC-YVAD-pNA and AC-VEID-pNA. Interestingly, no changes in the activities of caspase 1 and caspase 6 were found in oxidative stress-induced cell death between mutant APP-bearing PC12 cells and controls (data not shown).

**Increased Caspase 3 Activity of APPsw PC12 Cells in Response to Oxidative Stress**—Furthermore, we prepared extracts from H₂O₂-treated PC12 cells and measured their ability to cleave the colorimetric substrate to Ac-DEVD-pNA, which is specifically cleaved by caspase 3. Cellular extracts from untreated control cultures showed very low Ac-DEVD-pNA cleav-
age in all cell lines (Fig. 4). Under these base-line conditions, no significant differences could be detected between vector-, APPwt-, and APPsw-transfected PC12 cells. After exposure to oxidative stress, caspase 3 activity increased gradually and reached a maximum activity after 6 h of incubation, demonstrating a delayed increase as compared with the initiator caspases 2 and 8. APPsw and APPwt PC12 cells showed a significantly enhanced caspase 3 activity compared with vector-transfected clones with the strongest increase in the APPsw cells (Fig. 4A). By Western blotting, we also found a significantly increased protein expression of the active caspase 3 in APPsw PC12 cells compared with control cells (Fig. 4B). The highest protein expression was evidenced after 6 h of H$_2$O$_2$ exposure by densitometric immunoblot analysis (Fig. 4C).

**Activation of Caspase 9 in Response to Oxidative Stress**—Mitochondria play a prominent role in the intrinsic apoptosis pathway. During mitochondrial dysfunction, several essential players of apoptosis, including procaspases, cytochrome c, and apoptosis-inducing factor, are released into the cytosol. The multimeric complex formation of cytochrome c, apoptotic protease-activating factor-1, and caspase 9 activates downstream caspases, leading to apoptotic cell death (55). Therefore, we examined whether oxidative stress in PC12 cells activates caspase 9 by using an polyclonal antibody specific for the inactive full-length procaspase 9 and for the cleaved fragments of the protein. We detected a time-dependent increase in cleaved fragments of caspase 9 after treatment of the cells with hydrogen peroxide (Fig. 5A). APPsw PC12 cells showed an elevated

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**Figure 2**

**A**. Time course of caspase 2 activity in protein extracts from PC12 cells after treatment with H$_2$O$_2$. Over the whole time period, APPsw-bearing cells exhibit a significantly increased caspase 2 activity compared with APPwt-transfected cells and vector controls; two-way ANOVA revealed significant differences between cell lines ($p < 0.001$) and time course ($p < 0.001$). The caspase 2 activity was induced early in APPsw cells within 2 h of oxidative stress induction and 4-fold higher compared with APPwt and vector (ANOVA). Data are means ± S.E. from 4–6 independent experiments. **B**, representative Western blot analysis of caspase 2 activation in cell lysates of H$_2$O$_2$-treated PC12 cells. Increased expression of cleaved caspase 2 in APPsw cells is already detectable after 2 h of treatment.

**Figure 3**

A. Time course of caspase 8 activity in protein extracts from PC12 cells after treatment with H$_2$O$_2$. The maximal caspase 8 activity was measured after a 2-h induction. APPsw-bearing cells exhibit a significantly enhanced activity compared with vector controls ($^*, p < 0.05$ versus vector (ANOVA)). **B**, the maximal caspase 8 activity was reached after 2 h of induction, and 4-fold higher compared with APPwt and vector controls ($^{**}, p < 0.01$ versus APPwt and vector (ANOVA)). **C**, over the whole time period, the protein expression in APPsw cells was continuously higher compared with vector control as detected by an antibody recognizing the 26-kDa large cleaved fragment of caspase 8. Representative Western blotting is shown from at least three independent observations.
expression of the active cleaved fragments of caspase 9 compared with vector-transfected cells. A time-dependent cytochrome c increase in the cytosol was also detected by Western blot using a specific antibody (Fig. 5D). The highest cytochrome c amount in the cytosolic fraction was detected after a 4-h oxidative stress induction. In addition, caspase 9 activities were measured colorimetrically using Ac-LEHD-pNA as substrate in lysates of hydrogen peroxide-treated cells (Fig. 5B).

We ascertained an early induced activation of caspase 9 after 2 h, whereas the activity was increased in the APP-transfected cells compared with vector-transfected controls but was not different between APPsw and APPwt cells (Fig. 5C).

Enhanced Activation of JNK Pathway in APPsw PC12 Cells—The mobilization of the stress-activated protein kinase (JNK) plays an important role in apoptosis induced by several types of environmental stress, like UV, γ-radiation, inflammatory cytokines, and DNA damage (56–58). In oxidative stress-induced mitochondrial apoptosis machinery by JNK in adult cardiac myocytes has been observed (37). Hence, we addressed the question whether oxidative stress-mediated activation of the JNK pathway is affected by overexpression of APP. We performed a Western blotting of immunocomplexes to detect activation of JNK and c-Jun. H2O2 treatment of APPsw PC12 cells induced JNK phosphorylation within 2 h (Fig. 6A). The highest expression was detected after 4 h with an elevated expression in APPsw cells compared with APPwt cells and vector control. When activated, JNK translocates to the nucleus to regulate transcription through its effects on c-Jun and other transcription factors. As for JNK, we observed a strong impact of the Swedish APP transfection on the activation of c-Jun (Fig. 6B). In our experiments, the kinetics of activation of c-Jun correlated with the phosphorylation of JNK with the highest detection of phosphorylated c-Jun after 6 h of H2O2 treatment. The expression of phosphorylated c-Jun was markedly elevated in APPsw compared with treated APPwt or vector-transfected controls after 4 and 6 h of hydrogen peroxide treatment.

Prevention of Apoptosis by Caspase Inhibitors—Due to the enhanced activation of several caspases in response to oxidative stress, we examined the potential against several caspase inhibitors in protecting PC12 cells of oxidative stress-induced apoptosis. Caspase inhibitors were added to cultures of PC12 cells in a time- and concentration-dependent manner (data not shown). Best protection was provided by adding caspase inhibitors at a concentration of 10 μM h prior to H2O2 exposure. As shown in Fig. 7, only the inhibitor of the effector caspase 3, AC-DEV-DMK, was able to rescue APPsw cells from apoptosis below the level of that from vector-transfected control cells. The pretreatment of the APPsw cells with inhibitors of initiator caspase 8 and caspase 9 revealed similar levels than did vector cells.

Inhibition of JNK by SP600125—As detected by Western blotting, we could show that the transfection of the Swedish FAD mutation in PC 12 cells leads to an enhanced activation of the JNK pathway under oxidative stress. Therefore, we investigated the effect of SP600125, a selective antrapyrazolone inhibitor of JNK (59), in our cell model. We measured the caspase activities of different caspases and the mitochondrial membrane potential after pretreatment with JNK inhibitor to attribute the JNK involvement in oxidative stress-induced apoptosis. We found a highly significant reduction of caspase 9 activity (two-way ANOVA, p < 0.001) and caspase 3 activity (two-way ANOVA, p = 0.0384) in cell lysates of APPsw cells pretreated with 200 μM JNK inhibitor, whereas the activities of caspase 2 and caspase 8 were not significantly altered (Fig. 8). Corresponding to the reduction of caspase 9 activity, we were able to detect a protection against a decrease in the mitochondrial membrane potential by SP600125 (Fig. 9), indicating an involvement of JNK upstream of mitochondria in oxidative stress signaling. Whereas the treatment with H2O2 (250 μM) led to a significant increase of apoptotic cells in APPsw-bearing PC12 cells (42.21 ± 3.71%) compared with vector cell line (30.87 ± 5.97%), APPsw cells pretreated with the JNK inhibitor (33.77 ± 4.06%) exhibited similar apoptotic cell levels as the corresponding vector cells (Fig. 7).

DISCUSSION

In this study, we provide evidence for the enhanced cell death vulnerability caused by the Swedish APP mutation and elucidate cell death mechanism probably initiated by intracellularly produced Aβ. Although Aβ-induced cell death has been a topic of research in many studies, the relevance of these in vitro findings with regard to in vivo processes is not clear. In most studies, synthetic Aβ peptides have been applied at micromolar levels that are in contrast to the low nanomolar levels of natural Aβ found in the brain and cerebrospinal fluid of AD patients.

Therefore, studies of Aβ-induced neurotoxic effects under more likely physiological conditions mimicking the situation in AD brains are needed (60). Moreover, Aβ is produced intracellularly and can accumulate within cells (21, 61, 62) before its deposition in senile plaques. Intracellular accumulation of Aβ might impair cellular functions and may represent the primary event within the neurotoxic Aβ cascade. In order to mimic the situation in vivo, we decided to transfect PC12 cells with human APP and the FAD-related Swedish APP mutation. APPsw and APPwt PC12 cells showed a moderate expression of human APP, leading to low levels of Aβ1–40 production (APPsw, 90 pg/ml; APPwt, 20 pg/ml) close to the situation in vivo. These two cell lines with different production levels of Aβ may addi-
tionally allow us to study dose-dependent effects of Aβ. Expression of APPsw rendered PC12 cells more vulnerable to the induction of cell death after exposure to oxidative stress. It seems likely that increased production of Aβ at physiological levels primes APPsw cells to undergo cell death only after additional stress, a scenario that has been suggested to occur in AD brain. Moreover, we were able to show that caspase 3 activity was significantly enhanced in APPsw compared with APPwt and vector-transfected cells. Interestingly, APPwt also showed an increased activity of caspase 3 compared with empty

![Figure 5](image)

**FIG. 5.** A, Western blot analysis of caspase 9 activation in cell lysates of H2O2-treated PC12 cells. Data are from a representative experiment repeated at least three times with similar findings. Increased expression of cleaved caspase 9 in APPsw cells is already detectable after 2 h of treatment. B and C, enhanced caspase 9 activity in cell lysates from APP-transfected cells compared with vector-transfected controls (\(^*\), \(p < 0.05\) versus vector after 2 h of induction; \(\dagger\), \(p < 0.05\) versus vector and wild type under basal conditions). C, cytochrome c release from the mitochondria. Cytoplasmic fractions were separated from mitochondrion-enriched fractions and subjected to Western blotting. A time-dependent cytochrome c increase in the cytosol was detected. As a control, the blot was probed with an actin antibody to evaluate the loading of the cytosolic extracts.

![Figure 6](image)

**FIG. 6.** Activation of JNK and c-Jun in oxidative stress-induced cell death in APPsw-, APPwt-, and vector-transfected cells analyzed by Western blotting using anti-active p-JNK and p-c-Jun antibodies for the analysis of stress-activated protein kinase/JNK and c-Jun phosphorylation/activity. A, APPsw cells exhibit significantly enhanced protein expression levels of JNK activation in PC12 cells compared with APPwt and vector-transfected cells. Maximal activated phosphorylated JNK was detected after a 4-h exposure to oxidative stress. B, the expression of phosphorylated c-Jun was markedly elevated in APPsw cells compared with H2O2-treated APPwt and vector controls after hydrogen peroxide treatment. Maximal amount of phosphorylated c-Jun was detected after a 6-h H2O2 treatment of the cells. C, the blots were reprobed with an anti-actin antibody to confirm the equal loads of the protein amounts. Representative Western blotting are shown from at least three independent observations.

![Figure 7](image)

**FIG. 7.** Apoptotic cell death after treatment with H2O2 in PC12 cells. Apoptotic cell death was assessed by propidium iodide staining and flow cytometry. APPsw PC12 cells showed enhanced sensitivity to oxidative stress-induced death compared with vector cells (\(\dagger\), \(p < 0.05\); Student’s t test). In parallel experiments, APPsw cells were pretreated for 3 h with different caspase inhibitors (10 μM) or the JNK inhibitor SP600125 (200 nM) and then exposed to H2O2 for 24 h. The caspase 3 inhibitor strongly reduced apoptotic cell death in APPsw cells (\(\ast\ast\), \(p < 0.01\); paired t test). Pretreatment of APPsw cells with JNK, caspase 8, and caspase 9 inhibitors reduced apoptotic cell death to levels corresponding to that of vector cells. Values are means ± S.E. from 6–8 independent experiments.
vector control cells, although to a lesser extent than APPsw cells. Our data suggest that already very low Aβ levels in APPwt cells are probably sufficient to prime cells to undergo apoptosis. This is confirmed by our findings that caspase 9 activity is increased and at the same time mitochondrial membrane potential is lowered in APPwt as well as in APPsw cells compared with vector-transfected control cells after the induction of oxidative stress. In addition, we examined the implication of two other initiator caspases, which has been described in the literature.

**Fig. 8. Reduction of caspase activities by JNK-inhibitor SP60125.** Cells were pretreated with 200 nM SP600125 and incubated with H2O2 in a time-dependent manner. Caspase activities were measured in cytosolic protein extracts. Caspase 9 activity in cell lysates of APPsw cells pretreated with JNK inhibitor was significantly reduced compared with APPsw cells without pretreatment with the inhibitor (two-way ANOVA; p < 0.001 for pretreatment). The maximal reduction of caspase 9 activity by pretreatment of the cells with JNK inhibitor was measured after a 2-h exposure to oxidative stress (**, p < 0.01 versus corresponding pretreated cells; *, p < 0.05 versus corresponding pretreated cells after 4-h H2O2 induction). Caspase 3 was also reduced by the JNK inhibitor (two-way ANOVA; p < 0.01 for time course and pretreatment) with maximal reduction of caspase 3 activity after 2-h exposure to oxidative stress (*, p < 0.05 versus corresponding pretreated cells). The activities of caspase 2 and caspase 8 were not significantly altered. Values are means ± S.E. from 4–6 independent experiments.

**Fig. 9. Mitochondrial membrane potential analysis: Oxidative stress leads to a decrease in the mitochondrial membrane potential ($\Delta\Psi_m$) in PC12 cells.** The strongest reduction of the mitochondrial membrane potential occurs in APPsw cells (§§, p < 0.01 versus corresponding H2O2-treated vector cells; t test). The pretreatment of cells with SP600125 before exposure to H2O2 significantly protects the mitochondria against oxidative damage and decrease in $\Delta\Psi_m$ in all three cell lines (**, p < 0.01 versus corresponding H2O2-treated cells, paired t test).

**Fig. 10. Hypothetical sequence of events leading to cell death occurring in FAD.** Different pathways may occur in APP-transfected PC12 cells under oxidative stress conditions. The intrinsic pathway is activated in APPwt and APPsw bearing cells leading to mitochondrial dysfunction in an Aβ-dose-dependent manner compared with vector-transfected controls. Mitochondria may act as amplifiers, because cytochrome c is released and activates caspase 9. Since no differences in cytochrome c release and caspase 9 activity between APPsw and APPwt cells were observed, other proapoptotic factors from the mitochondria like Diablo/Smac followed by inhibition of members of the inhibitor-of-apoptosis (IAPs) family could be involved. The 4–5-fold elevated Aβ levels in APPsw compared with APPwt cells resulted in enhanced JNK, caspase 2, and caspase 8 activation. The convergence of all pathways results in activation of the caspase 3 and the execution of cell death. Thus, the blocking of initiator caspasess only inhibits the amplification loop and does not prevent cell death.
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primarily in the extrinsic apoptotic pathway. We demonstrated that caspase 8, involved in death receptor signaling, is activated in oxidative stress signaling in PC12 cells. Importantly, this activation is markedly elevated in APPsw cells compared with APPwt cells. Whether this increase is due to effects of extracellularly secreted Aβ on cell death receptors or is mediated by intracellularly accumulated Aβ is not clear and still under investigation. In parallel, we observed in PC12 cells an activation of caspase 2 in oxidative stress signaling. In APPsw cells, the maximal activity was 4-fold higher compared with APPwt and vector PC12 cells, respectively. This finding is of particular interest, since recent data describe caspase 2 to be localized in several intracellular compartments, including the mitochondria, Golgi, cytosol, and nucleus (63–65). Cytotoxic stress causes activation of caspase 2, which is required for the permeabilization of mitochondria (66) and induces the release of cytochrome C (64, 67, 68). It is assumable that the enhanced Aβ production in the APPsw-transfected cells occurs through an increased expression and activity of beta-site APP-cleaving enzyme (26). The elevated intracellular levels of Aβ in the mutant cells may therefore lead to an enhanced activation of caspase 2, which in turn implements the activation of the intrinsic apoptotic pathway by permeabilizing mitochondria (69). As a consequence, cytochrome c is released to the cytosol, resulting in an activation of the apoptotic protease-activating factor-1/caspase 9 apoptosisome complex. Simultaneously, other effects of caspase 2 need to be considered, since we determined no differences in cytochrome C release and caspase 9 activation between APPsw and APPwt cells. Recent data indicate that Aβ induces the release of other proapoptotic factors from the mitochondria (e.g. Diablo/Smac (second mitochondria-derived activator of caspase)) (70). Thus, Smac or other unknown factors may be involved in our cell model. Since caspase 2 activation occurs early in our cell model and neurons from caspase 2 null mice are resistant to β-amyloid-induced death (47), this caspase seems to play a critical role.

Given the important role that mitogen-activated protein kinase pathways play in cellular stress signaling, we investigated the relationship between activated JNK, apoptosis, and the Swedish APP mutation. We demonstrated that oxidative stress leads to an activation of JNK. JNK phosphorylates specific sites in the N-terminal region of c-Jun, thereby enhancing its transcriptional activity. Very importantly, we were able to demonstrate that in PC12 cells bearing mutant APPsw, the activation of JNK and c-Jun was significantly enhanced compared with APPwt cells. At the same time, activation of JNK and c-Jun is increased in APPwt compared with vector cells. This suggests that the different Aβ levels induce a dose-dependent activation of JNK pathway. Interestingly, we could observe a reduction of caspase 9 activity using the selective JNK inhibitor SP600125, whereas activities of caspase 2 and caspase 8 were unaltered. Furthermore, the inhibitor compensated the mitochondrial abnormality when oxidative stress was induced. Due to the fact that members of the antiapoptotic Bcl-2 family proteins are inactivated through phosphorylation by JNK (71, 72), our results support evidence that JNK alters mitochondrial function. Recently, it was reported by different groups that JNK-interacting proteins JIP1b and JIP2 bind the cytoplasmic domain of APP (73–76). JIP family proteins are scaffold proteins that organize specific members of the JNK/mitogen-activated protein kinase cascade to facilitate signaling. It was proposed that these scaffold proteins could serve as cargo for the microtubule motor kinesin to mediate the transport of several transmembrane proteins (77), in which APP is supposed to be a cargo receptor (78). Our data indicate that the altered processing of APPsw might play a crucial role in activation of the stress kinase pathway. Whether this occurs by a redistribution of these scaffolds proteins has to be clarified in the future.

In addition, we investigated the protective effects of caspase inhibitors as well as JNK inhibitor in preventing oxidative-stress-mediated cell death. Only the caspase 3 inhibitor was able to reduce oxidative stress-induced apoptosis significantly in APPsw cells below the level of controls cells. The inhibition of caspase 8 or caspase 9 was less effective in preventing apoptosis. The lack of efficacy of the initial caspase inhibitors to abolish completely apoptotic cell death could be attributed to the complicated pathway. Although the treatment of PC12 cells with hydrogen peroxide leads to activation of these initiator caspases, their inhibition is not enough to avoid apoptosis, because different caspase pathways co-exist (Fig. 10). Such a compensation of caspase pathway was ascertained in sympathetic neurons from caspase 2 null animals. A lack in caspase 2 activity inactivation of JNK, apoptosis, and mitochondrial dysfunction with caspase pathway and neuronal loss for our cell model (Fig. 10). The brain has a high metabolic rate and is exposed to gradually rising levels of oxidative stress during life. In Swedish FAD patients, the levels of oxidative stress are increased in the temporal inferior cortex (28). Our study using a cell model mimicking the in vivo situation in AD brains indicates that probably both increased Aβ production and the gradual rise of oxidative stress throughout life lead to an increased vulnerability to apoptotic cell death in neurons from FAD patients.

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