Amyloid β-induced Changes in Nitric Oxide Production and Mitochondrial Activity Lead to Apoptosis*

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Increasing evidence suggests an important role of mitochondrial dysfunction in the pathogenesis of Alzheimer’s disease. Thus, we investigated the effects of acute and chronic exposure to increasing concentrations of amyloid β (Aβ) on mitochondrial function and nitric oxide (NO) production in vitro and in vivo. Our data demonstrate that PC12 cells and human embryonic kidney cells bearing the Swedish double mutation in the amyloid precursor protein gene (APPSw), exhibiting substantial Aβ levels, have increased NO levels and reduced ATP levels. The inhibition of intracellular Aβ production by a functional γ-secretase inhibitor normalizes NO and ATP levels, indicating a direct involvement of Aβ in these processes. Extracellular treatment of PC12 cells with comparable Aβ concentrations only leads to weak changes, demonstrating the important role of intracellular Aβ. In 3-month-old APP transgenic (tg) mice, which exhibit no plaques but already detectable Aβ levels in the brain, reduced ATP levels can also be observed showing the in vivo relevance of our findings. Moreover, we could demonstrate that APP is present in the mitochondria of APPSw PC12 cells. This presence might be directly involved in the impairment of cytochrome c oxidase activity and depletion of ATP levels in APPSw PC12 cells. In addition, APPSw human embryonic kidney cells, which produce 20-fold increased Aβ levels compared with APPSw PC12 cells, and APP tg mice already show a significantly decreased mitochondrial membrane potential under basal conditions. We suggest a hypothetical sequence of pathogenic steps linking mutant APP expression and amyloid production with enhanced NO production and mitochondrial dysfunction finally leading to cell death.

Alzheimer’s disease (AD)† is the most common neurodegenerative disorder (1) marked by progressive loss of memory and cognitive ability. The pathology of AD is characterized by the presence of amyloid plaques (2) and intracellular neurofibrillary tangles and pronounced cell death. The amyloid plaque is composed of amyloid β (Aβ) peptide (3), which is derived from the amyloid precursor protein (APP) through an initial β-secretase cleavage followed by an intramembranous cut of γ-secretase (4, 5). Autosomal dominant forms are caused by mutations in APP, presenilin 1, and presenilin 2, mainly associated with the early onset of AD (6). The Swedish double mutation in the APP gene (K670M/N671I) results in a 6–8-fold increased Aβ production compared with human wild type APP cells (APPwt) (7, 8). We have previously shown that the APPSw mutation enhances the vulnerability to secondary insults, e.g. oxidative stress, finally leading to apoptotic cell death through the activation of the c-Jun N-terminal kinase and caspases 3 and 9 (9). The latter observation provided evidence that mitochondria-mediated apoptosis might play an important role in these processes.

Intrinsic apoptotic pathway via mitochondria is regulated by members of the Bcl-2 family (10). They are mainly localized in the outer mitochondrial membrane. Bcl-2 and Bcl-xL inhibit apoptosis, whereas other members such as Bax, Bak, Bid, Bik, and Bim are proapoptotic (11). Their most frequently reported mode of action is the regulation of cytochrome c release from the intermembrane space (12). Once released from the mitochondrion, cytochrome c interacts with Apaf 1 and procaspase 9 to activate caspase 3, finally leading to cell death. Smac (second mitochondria-derived activator of caspase) is another mitochondrial intermembrane protein that is released in the cytosol during apoptosis (13). Smac interacts with several inhibitors of apoptosis, thereby relieving the inhibitory effect of inhibitors of apoptosis on caspases (14). As an early event in the apoptotic pathway, typically, a rapid reduction of the mitochondrial membrane potential (Ψm) takes place (15), which reflects a block of the respiratory chain finally leading to the reduction of ATP levels.

Some previous findings suggest that neurotoxicity of Aβ (16, 17) seems to be mediated via oxidative stress probably leading to mitochondrial damage, e.g. Aβ inhibited cytochrome c oxidase (COX) activity in isolated brain mitochondria (18, 19), and type APP; NO, nitric oxide; NOS, nitric-oxide synthase; ELISA, enzyme-linked immunosorbent assay; HEK, human embryonic kidney; tg, transgenic; COX, cytochrome c oxidase; ROS, reactive oxygen species; Ψm, mitochondrial membrane potential; Smac, second mitochondria-derived activator of caspase; AIF, apoptosis-inducing factor; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; TMRE, tetramethylrhodamine ester; ANOVA, analysis of variance; DAPT, N-[N-(3,5-trifluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyler ester.

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§§§ The abbreviations used are: AD, Alzheimer’s disease; Aβ, amyloid β peptide; APP, amyloid precursor protein; FAD, familial Alzheimer’s disease; APPSw, Swedish double mutation form of APP; APPwt, wild-type APP; NO, nitric oxide; NOS, nitric oxide synthase; ELISA, enzyme-linked immunosorbent assay; HEK, human embryonic kidney; tg, transgenic; COX, cytochrome c oxidase; ROS, reactive oxygen species; Ψm, mitochondrial membrane potential; Smac, second mitochondria-derived activator of caspase; AIF, apoptosis-inducing factor; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; TMRE, tetramethylrhodamine ester; ANOVA, analysis of variance; DAPT, N-[N-(3,5-trifluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyler ester.
in neuronal cultures, Aβ caused a loss of activity of all mitochondrial respiratory chain complexes (20–23). Moreover, activity changes in mitochondrial enzymes including pyruvate dehydrogenase and α-ketoglutarate dehydrogenase have been described in AD brain (24). In addition, patients with AD showed impaired cytochrome c oxidase activity in the central nervous system and even in other tissues including platelets (19, 25–27). Interestingly, p2 cells repopulated with mitochondria from AD patients also showed a reduced cytochrome c oxidase activity (28). Thus, the defects in mitochondrial energy metabolism that lead to increased production of reactive oxygen species (ROS) seem to underlie the pathology of AD (29). Mitochondria represent not only the major source of ROS but also the main target for ROS damage. Nitric oxide (NO) and its derivative (reactive nitrogen species), also belonging to the group of ROS, are known to inhibit the mitochondrial respiration (30). Hereby, NO itself causes a selective reversible inhibition of cytochrome c oxidase (31), whereas RNS inactivate multiple respiratory chain complexes and ATP synthase (32). In accordance, both oxidative and nitrosative stress seem to represent early events in the pathogenesis of AD (33–36).

On the basis of these findings, we set out to investigate the precise mechanisms underlying the action of Aβ and/or expression of mutant APP on mitochondrial function in multiple experimental designs mimicking different in vitro situations that are discussed to occur in AD patients: 1) in cell lines overexpressing different levels of Aβ (APPsw PC12 cells exhibiting low physiological concentrations of Aβ within picomolar range and APPsw HEK cells expressing Aβ levels within low nanomolar range) to study dose-dependent effects of Aβ in an in vitro setting characterized by chronic Aβ production due to increased APP processing; 2) after extracellular Aβ treatment to distinguish chronic from acute and/or extracellular actions from effects of intracellular Aβ or APP-processing products on mitochondria; and 3) in a secondary insult model to examine the additional impact of oxidative stress. Finally, we checked the in vivo relevance of our in vitro findings by studying mitochondrial function in brain cells from APP transgenic (tg) mice.

**EXPERIMENTAL PROCEDURES**

**Materials**—Rhodamine 123 and tetramethylrhodamineethyl ester (TMRE) were purchased from Molecular Probes. 4,5-Diaminofluorescein diacetate was obtained from Calbiochem. ViaLight HT kit was purchased from Cambrex. Cytochrome c oxidase assay kit, hydrogen peroxide, rotenone, thenoyltrifluoroacetone, antimycin, sodium azide (NaN3), and oligomycin were obtained from Sigma. The rabbit anti-human Abeta1–40 C-terminal detection antibody (site-specific rabbit anti-human Abeta1–40) was obtained from Biotrend. Aβ was supplied by Bachem. DAPT was obtained from Merck Biosciences.

**Cell Culture and Transfection**—PC12 cells and HEK cells were transfected with DNA constructs harboring human mutant APP (APPsw, K670M/N671L) gene, the APPwt gene, inserted downstream of a cytomegalovirus promoter using the FUGENE 6 technique (Roche Diagnostics) (37). The transfected cells APPwt PC12, APPsw PC12, and control PC12 were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum and 5% heat-inactivated horse serum, 50 units/ml penicillin, 50 μg/ml streptomycin, and 400 μg/ml G418 at 37 °C in a humidified incubator containing 5% CO2.

The transfected cell lines APPwt HEK, APPsw HEK, and control HEK were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum, 50 units/ml penicillin, 50 μg/ml streptomycin, and 400 μg/ml G418 at 37 °C in a humidified incubator containing 5% CO2. The Aβ levels of APP-transfected PC12 cells and HEK cells are shown in Table I.

**Detection of Aβ Levels**—For the detection of secreted Aβ1–42, a specific sandwich enzyme-linked immunosorbent assay (ELISA) employing monoclonal antibodies was used. The ELISA was performed according to the instructions given in the Abeta-ELISA kit by BIOSOURCE. The assay principle is that of a standard sandwich ELISA, which utilizes a monoclonal mouse anti-human Abeta1–18 capture antibody, a cleavage site-specific rabbit anti-human Abeta1–40 C-terminal detection antibody, and anti-rabbit IgG peroxidase-conjugated secondary antibody.

**Transgenic Animal Brain Tissue**—Female C57BL/6 mice bearing the human Swedish and London mutations in the 751 amino acid form of human amyloid precursor protein (tg APP) under the control of a murine Thy-1 promoter at an age of 3 months and non-transgenic littermate animals were used for the experiments (38). ABAPP mice tgs mice exhibited the onset of Aβ plaques at an age of 4–6 months, but intracellular Aβ load was already detectable at the age of 3 months (39).

Mice were sacrificed by decapitation, and brains were quickly dissected on ice (method modified after Stoll et al. (40)). After removing the cerebellum, the tissue was minced into 2 ml of medium I (138 NaCl, 5.4 KCl, 0.17 Na2HPO4, 0.22 K2PO4, 5.5 glucose, and 58.4 sucrate, all in mol/liter, pH 7.4) by centrifugation (400 × g for 3 min at 4 °C). 100 μl of the suspension was used for protein determination. After centrifugation, cells were resuspended in 6 ml of Dulbecco’s modified Eagle’s medium and 500 μl/well were distributed on a 24-well plate for the measurement of mitochondrial membrane potential. For the measurement of ATP levels, 100 μl/well were distributed on a white 96-well plate. The preparations of APP tg mice and non-transgenic littermate mice (overcross design) were made under the same conditions and in parallel.

**Quantification of Apoptosis by Flow Cytometry**—Apoptosis was determined by propidium iodide staining and fluorescence-activated cell sorter analysis as described previously (37). PC12 cells and HEK cells were lysed in buffer (0.1% sodium citrate and 0.1% Triton X-100) containing 50 μg/ml propidium iodide. Samples were analyzed by flow cytometry (FACS Calibur) using Cell Quest software (BD Biosciences). Cells with a lower DNA content showing less propidium iodide staining than G1 have been defined as apoptotic cells (sub-G1 peak).

**Determination of Intracellular Nitric Oxide Levels**—PC12 cells and HEK cells were plated the day before at a density of 2 × 103 cells/well in a 24-well plate. To measure the NO levels, the fluorescence dye 4,5-diaminofluorescein diacetate was used in a concentration of 10 μM for 30 min (41). The cells were washed twice with Hank’s balanced salt solution, and the fluorescence was determined with a fluorescence reader (Victor® multilabel counter) at 490/535 nm.

NO levels were also determined after a 4-h incubation in the absence or in the presence of the NO synthase inhibitors 20 μM L-NAME, 1 mM N-propyl-L-arginine, and 1 mM 1400W.

**Determination of the Mitochondrial Membrane Potential (ϕm)**—PC12 cells and HEK cells were plated the day before at a density of 2 × 105 cells/well in a 24-well plate. PC12 cells were incubated with H2O2 (0.5 mM) for different periods of time. The mitochondrial membrane potential was measured using the fluorescence dye Rhodamine 123 (42). Transmembrane distribution of the dye depends on the mitochondrial membrane potential. The dye was added to the cell culture medium in a concentration of 0.4 μM for 15 min. The cells were washed twice with Hank’s balanced salt solution, and the fluorescence was determined with a fluorescence reader (Victor multilabel counter) at 490/535 nm.

The mitochondrial membrane potential of dissociated neurons was also measured with Rhodamine 123 in a concentration of 0.4 μM for 15 min. For detailed information regarding the preparation, see “Transgenic Animal Brain Tissue.”

To test acute and fast changes in ϕm, the fluorescence dye TMRE (43) was used in a concentration of 0.4 μM for 15 min. TMRE exhibits a characteristic increase in fluorescence at 490/590 nm after challenging mitochondria with membrane potential-decreasing drugs (44). The mitochondrial membrane potential was recorded, and then the complex inhibitors (2 μM rotenone, 10 μM thenoyltrifluoroacetone, 2 μM antimycin, 10 μM oligomycin, and 10 mM sodium azide) were added.

**Determination of ATP Levels with a Bioluminescence Assay**—PC12 cells and HEK cells were plated the day before at a density of 2 × 105 cells/well in a white 96-well plate. PC12 cells were incubated for different periods of time with H2O2 (0.1 mM).

The ATP levels of dissociated neurons were also measured with the bioluminescence assay. For detailed information regarding this preparation, see “Transgenic Animal Brain Tissue.”

The kit is based upon the bioluminescent measurement of ATP (45).
digitonin. Following a centrifugation step at 800 ×g, methylsulfonyl fluoride, additional protease inhibitors, and 0.05% SDS were used. Difluoride membranes then were exposed to the following antibodies: mouse anti-human APP W02 (Abeta; W02 antibody is directed against the C-terminal sequence of the 1–42 peptide); rabbit anti-calreticulin (Chemicon); mouse anti-Na+/K+-ATPase (Chemicon); rabbit anti-nitrotyrosine (Merck Biosciences); and rabbit anti-cytochrome c oxidase (Merck Biosciences). The protein bands were detected by ECL reagent (Amersham Biosciences). After detection, the membranes were treated with stripping buffer (100 mM glycine, pH 2.5) for 2 h after reprobing with different antibodies.

**Isolation of Cytosolic and Mitochondrial Fractions**—Cytosolic and mitochondrial fractions were isolated by digitonin permeabilization (46). 5 × 10^6 cells were washed with ice-cold phosphate-buffered saline, and cells were resuspended for 15 min on ice in permeabilization buffer containing 75 mM NaCl, 1 mM NaH_2PO_4, 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 separated from the pellet consisting of cellular debris. The crude mitochondrial pellet was collected by centrifugation at 13,000 × g at 4 °C for 10 min. The supernatant containing cytoplasmic proteins was stored at −20 °C for further investigation. The pellet containing mitochondria was dissolved in phosphate-buffered saline for the cytochrome c oxidase assay and resuspended in 0.1% Triton X-100 and mechanically lysed for the Western blot. The total protein content was determined by the method of Lowry (Bio-Rad).

**Detection of Cytochrome c Oxidase Activity in Isolated Mitochondria with Cytochrome c Oxidase Assay Kit (47)**—The colorimetric assay is based on the observation of the decrease in absorbance at 550 nm of ferrocyanochrome c caused by its oxidation to ferricytochrome c by cytochrome c oxidase. The cytochrome c oxidase assay was performed according to the instructions given in the kit.

**Western Blot**—After determination of the total protein content by the method of Lowry, the cytosolic and mitochondrial fraction was mixed with 4% Laemmli sample buffer and denatured for 10 min at 95 °C. An equal amount (10–20 μg) of protein was loaded per lane on acrylamide gels and examined by SDS-PAGE. The cytochrome b_6f complex was separated to determine the protein composition of the mitochondrial fraction.

**RESULTS**

**APP Expression and Aβ Levels of APP-transfected PC12 Cells and HEK Cells**—Increasing knowledge was obtained that Aβ is produced intracellularly (48) and that intraneuronal accumulation of Aβ precedes plaque formation in APP tg mice bearing the Swedish double mutation (49, 50). Thus, intracellular accumulation of Aβ might be a primary step in the neurotoxicity cascade of Aβ in vivo and in vitro. APP containing the Swedish mutation resulted in an overall increase of Aβ including both Aβ_1–40 and Aβ_1–42.

In our cell model, the APPsw mutation resulted in a markedly increased Aβ secretion of PC12 cells (0.20 nmol/liter) as well as in HEK cells (5.42 nmol/liter) compared with APPwt PC12 cells and APPwt HEK cells. APPsw PC12 cells secreted low Aβ levels, reflecting the physiological situation in vivo during cellular metabolism (7), whereas human APP was equally expressed in APPwt and APPsw PC12 cells and was increased 2-fold compared with the endogenous APP expression of control PC12 cells (37). Of note, HEK cells bearing the APPsw mutation exhibited an ∼20-fold increased Aβ secretion compared with APPsw-bearing PC12 cells (Table 1), a scenario that might occur in familial AD and that could explain the more drastic effects in some assay-designs. Furthermore, this might indicate that, in APPsw HEK cells, the secretion pathway is more pronounced than in PC12 cells. The endogenous human APP expression of control HEK cells was increased 2-fold compared with APPsw PC12 cells. Moreover, the APP expression of APPwt and APPsw HEK cells was increased 3-fold compared with control HEK cells and 6-fold compared with APPsw PC12 cells. Using the different cell lines allowed us to study the dose-dependent effects of Aβ on those stably transfected cells with APPwt or APPsw, both representing a model of chronic AD stress.

**Basal Apoptosis Was Increased in APPsw HEK Cells but Not in APPsw PC12 Cells**—Apoptotic cell death plays a very important role in the pathogenesis of AD (51). Thus, we investigated basal apoptosis using propidium iodide staining. In APPsw PC12 cells, we observed no increased basal apoptosis (Fig. 1A). Thus, APPsw PC12 cells were able to compensate for the consequences of the increased Aβ levels. However, APPsw HEK cells, which have high Aβ levels, showed significantly increased levels of apoptotic cells under base-line conditions (Fig. 1B). Additionally, the treatment of PC12 cells with extracellular Aβ_1–42 at concentrations of 10 nm and higher resulted also in increased apoptotic cell death (Fig. 1C). The effect on the induction of apoptosis was less pronounced after extracellular Aβ treatment than in APPsw HEK cells. Even the Aβ_1–42 peptide was used, which is more toxic than the Aβ_1–40 peptide.

**NO Levels Increased in APPsw Cells**—Excessive generation of nitric oxide has been implicated in the pathogenesis of AD (52). Thus, we measured intracellular NO levels using the fluorescence dye 4,5-diaminofluorescein diacetate in APP-transfected PC12 cells and HEK cells. Our data demonstrated that NO levels are significantly enhanced in the following order: control cells < APPwt < APPsw (Fig. 1, E and F) in both cell lines at a similar level. To determine the contribution of extracellular Aβ on NO levels, we treated PC12 cells extra-
cellularly with Aβ₁₋₄₂ (Fig. 1G). Interestingly, NO levels were also enhanced after exposure to Aβ₁₋₄₂ with a maximal effect already at 1 nM but to a lesser extent than in APPsw PC12 cells. Thus, the extracellularly applied Aβ cannot completely mimic the physiological situation of APPsw transfection.

NO Synthase (NOS) Activity Was Enhanced in APPsw Cells—NO was synthesized from L-arginine by three isoforms of NOS, two of which (endothelial NOS and neuronal NOS) were constitutively expressed, whereas the third (inducible NOS) was induced during inflammation (53). There may also be a mitochondrial isoform (54–56). To study NOS activity, we used L-NAME, an unselective NOS inhibitor. Interestingly, L-NAME was able to reduce the NO levels of PC12 cells. NOS activity is enhanced in APPsw PC12 cells. NO levels of APP-transfected PC12 cells were determined after a 48-h incubation in the absence or in the presence of the NO synthase inhibitor L-NAME (20 mM) (ANOVA: *** p < 0.001 versus control PC12 cells; ***, p < 0.001; ** p < 0.01 versus untreated APPwt and APPsw PC12 cells). Values are means ± S.E. from 6–8 experiments.

Mitochondrial membrane potential has been related to cell death in different cell types. Interestingly, APPsw-bearing cells showed a hyperpolarized mitochondrial membrane potential compared with control cells and APPwt PC12 cells.
extracellular Aβ (HEK cells are significantly decreased (ANOVA: ***,
S.E. from 9–18 experiments. p***, reduced ATP levels (Fig. 2A).

In addition, the extracellular Aβ-treatment for 24 h leads to a reduction of ATP levels in APPsw HEK cells, which secreted 20-fold increased Aβ levels after 48 h in APPsw HEK cells (Table I). Thus, the reduction of intracellular Aβ levels without affecting APP expression (59–61). In PC12 and HEK cells, DAPT does not show neurotoxicity by itself. We observed no decrease in MTT reduction after 24, 48, and 72 h of DAPT incubation (data not shown). However, we could show a strong reduction of secreted Aβ1–40 levels already after 24 h of DAPT incubation in APPsw PC12 cells and after 48 h in APPsw HEK cells (Table I). Thus, the reduction of secreted Aβ1–40 below the level of control cells was very drastic in APPsw HEK cells, which secreted 20-fold increased Aβ levels compared with APPsw PC12 cells. A similar decrease in secreted Aβ was also seen in APPwt cells (data not shown). As already shown by others (62), DAPT also reduces significantly grades of mitochondrial damage is due to intracellular Aβ and not due to overexpression of APP, APP-transfected PC12 and HEK cells were treated with DAPT, a functional γ-secretase inhibitor that reduces intracellular Aβ levels without affecting APP expression (59–61). In PC12 and HEK cells, DAPT does not show neurotoxicity by itself. We observed no decrease in MTT reduction after 24, 48, and 72 h of DAPT incubation (data not shown). However, we could show a strong reduction of secreted Aβ1–40 levels already after 24 h of DAPT incubation in APPsw PC12 cells and after 48 h in APPsw HEK cells (Table I). Thus, the reduction of secreted Aβ1–40 below the level of control cells was very drastic in APPsw HEK cells, which secreted 20-fold increased Aβ levels compared with APPsw PC12 cells. A similar decrease in secreted Aβ was also seen in APPwt cells (data not shown). As already shown by others (62), DAPT also reduces significantly
intracellular Aβ production in both cell lines. Of note, the reduction of Aβ levels by the γ-secretase inhibitor DAPT lead to a normalization of NO (Fig. 3A) and ATP levels (Fig. 3B) as well as mitochondrial membrane potential (Fig. 3C) in APPwt and APPsw PC12 and HEK cells, respectively. Thus, the increased NO production and mitochondrial damage in APP-transfected cells were triggered by the production and accumulation of Aβ.

Our data suggested that mainly the PC12 cell model represents a very suitable approach to elucidate AD-specific cell death pathways under physiological conditions that are the most relevant for the in vivo situation in man because PC12 cells bearing the APPsw mutation secrete Aβ levels within the picomolar range. For this reason, we made all of the following experiments with this cell model.

**APPsw PC12 Cells Have an Increased Amount of Mitochondria and Show Presence of APP in Mitochondria**—To exclude the possibility that ATP levels are reduced in APP-transfected PC12 cells due to a loss of mitochondria, we determined the amount of mitochondria in APP-transfected cells using the mitochondrial dye Mitotracker Red and confirmed co-localization with the mitochondrial membrane potential dye Rhodamine 123 (Fig. 4, A–C). Mitotracker Red selectively stains mitochondria independently from the mitochondrial membrane potential. Interestingly, APPsw PC12 cells contained more mitochondria than APPwt and control PC12 cells (Fig. 4, D–G). Thus, the drop of ATP levels in APPsw PC12 cells was due to mitochondrial dysfunction and not due to the loss of mitochondria. Moreover, the up-regulation of mitochondria seems not to be able to compensate for mitochondrial dysfunction with regard to ATP levels.

Recently, it has been shown that APP is also targeted to mitochondria (63) in addition to its localization in the endoplasmic reticulum and the plasma membrane. In this study, transiently transfected cells were used (63). Interestingly, we also observed the presence of APP in mitochondria of stably transfected APPwt and APPsw PC12 cells (Fig. 5). The purity of subcellular fractions was established by Western blot analysis of various fractions using antibodies to Na⁺/K⁺-ATPase (plasma membrane-specific), cytochrome c oxidase subunit 4 (mitochondria-specific), and calreticulin (endoplasmic reticulum-specific) as markers. Further investigations have to be done to further elucidate the role of APP in the mitochondrion.

**PC12 Cells Bearing the Swedish APP Mutation Showed an Impairment of the Mitochondrial Respiratory Chain**—NO is known to inhibit the mitochondrial respiratory chain (30). Therefore, we analyzed the COX activity. Interestingly, APPsw PC12 cells showed a significantly reduced cytochrome c oxidase activity compared with APPwt and control PC12 cells (Fig. 6A). Moreover, we measured the mitochondrial membrane potential with the fluorescence dye TMRE after stimulation with different complex inhibitors. Here, we showed that respiratory chain complexes II, III, IV, and F$_0$F$_1$-ATPase in APPsw PC12 cells are more vulnerable against mitochondrial membrane potential changes than control PC12 cells, indicating an impaired mitochondrial respiratory chain (Table II). Additionally, the complexes IV and F$_0$F$_1$-ATPase of APPwt PC12 cells are more sensitive than control PC12 cells but are as sensitive as APPsw PC12 cells. Thus, these two enzyme complexes are especially vulnerable to already very low Aβ concentrations.

**Oxidative Stress Induces Mitochondrial Damage in PC12 Cells Bearing the Swedish APP Mutation**—Oxidative stress might be involved in the pathogenesis of Alzheimer’s disease (64). Thus, we investigated the effect of a secondary insult, hydrogen peroxide, which increases cell death in APPsw PC12 cells (9), on mitochondrial membrane potential and ATP levels. Interestingly, APPsw-bearing PC12 cells showed a significantly decreased mitochondrial membrane potential after exposition to hydrogen peroxide in comparison with APPwt and control PC12 cells (Fig. 6B). Moreover, the ATP reduction after hydrogen peroxide exposure was more pronounced in APPsw and APPwt PC12 cells compared with control PC12 cells, even in the recovery phase during 2, 4, and 6 h of H$_2$O$_2$ exposure in a time-dependent manner (Fig. 6C). Thus, mutant cells have reduced capability to maintain mitochondrial membrane potential and ATP levels after oxidative stress and constantly showed an ATP deficiency.

**The Impact of Mutant APP on Members of the Bcl-2 Family and on the Release of Mitochondrial Factors**—The Bcl-2 family proteins are important regulators of apoptosis (10). They are proapoptotic or antiapoptotic in nature. That means that they induce or inhibit the release of mitochondrial proteins in the cytosol including cytochrome c, Smac, and AIF. By Western blot analysis, we investigated the proteins of the Bcl-2 family as well as Smac and AIF. We have previously shown that a time-dependent release of cytochrome c was observed after treatment with hydrogen peroxide, reaching a maximum after 6 h (9). After 4 h of treatment with hydrogen peroxide, there
was an enhanced release of Smac observed in APPsw PC12 cells (Fig. 7). AIF, a protein that causes nuclear condensation and DNA fragmentation, was not released by the mitochondria after 6 h of hydrogen peroxide incubation (Fig. 7); however, we observed a higher AIF expression in the mitochondria of APPsw PC12 cells at this time point. Interestingly, we found

**FIG. 4.** Staining of mitochondria in APP-transfected PC12 cells. A, APPsw PC12 cells were stained with Mitotracker Red. B, the same cell was co-stained with Rhodamine 123. C, co-localization of Mitotracker Red and Rhodamine 123. D, control PC12 cells. E, APPwt PC12 cells. F, APPsw PC12 cells were stained with Mitotracker Red. G, Mitotracker Red fluorescence of APPsw PC12 cells is enhanced. APPsw PC12 cells tend to have more mitochondria than APPwt and control PC12 cells (unpaired Student’s t test: *, p < 0.05 versus control PC12 cells). Values are means ± S.E. from six experiments.

**FIG. 5.** Western blot analysis of human APP expression in mitochondria of transfected PC12 cells. A, Western blot analysis of APP (arrows indicate mature and immature human APP695), actin, and COX4 in mitochondrial and cytosolic fraction. B, Western blot analysis of Na⁺/K⁺-ATPase and calreticulin in mitochondrial fraction and pellet. The pellet contains all of the cellular compartments with the exception of mitochondrial and cytosolic fraction. APP is present in mitochondria of APPwt and APPsw PC12 cells. The purity of subcellular fractions was evaluated by Western blot analysis of various subcellular fractions using antibodies to Na⁺/K⁺-ATPase (plasma membrane-specific), COX4 (mitochondria-specific), and calreticulin (endoplasmic reticulum-specific) as markers. Actin serves as a marker for equal loading.
AIF release after 24 h of hydrogen peroxide incubation (data not shown).

Concerning the Bcl-2 family, we found a reduced amount of the cytosolic anti-apoptotic protein Bcl-xL in APPsw cells under baseline conditions and after exposure to hydrogen peroxide but only minor changes in Bax protein content (Fig. 7). Thus, the Bcl-xL/Bax ratio was decreased in APPsw PC12 cells under basal conditions (control cells, 1.0; APPwt cells, 0.86; APPsw cells, 0.49), which might possibly explain the enhanced vulnerability to cell death after oxidative stress.

APP tg Mice Showed a Decreased Mitochondrial Membrane Potential and Reduced ATP Levels—To understand the in vivo relevance of our in vitro findings, we analyzed mitochondrial membrane potential and ATP levels in dissociated neurons of 3-month-old APP tg mice. Interestingly, the mitochondrial membrane potential in APP tg mice was significantly decreased under basal conditions compared with littermate non-tg control mice (Fig. 8). Additionally, dissociated neurons of tg APP mice showed reduced basal ATP levels (Fig. 8).

**DISCUSSION**

AD is associated with multiple lesions including changes in energy metabolism and altered mitochondrial structure and/or function in the brain. However, the precise mechanisms of mitochondrial pathology in AD are not clear.

Our study clearly demonstrates that Aβ induces mitochondrial adaptation and failure in a very vulnerable and dose-dependent pattern, which additionally overreacts to secondary insult, and that NO plays an important role in these processes. Several other studies already indicate that Aβ impairs mitochondrial function (18, 20, 65). However, nearly all of the studies used synthetic Aβ peptides in the micromolar range, many orders of magnitude over physiological levels, and cells or even isolated mitochondria were exposed only to extracellular Aβ. By contrast, our PC12 cell model represents a very valuable approach to investigate AD-specific cell death pathways by studying Aβ levels within the high picomolar range (7). This cell model attempts to mimic physiological conditions in man that are relevant for sporadic AD patients (66, 67). The comparison between transfected PC12 cells and HEK cells that exhibit Aβ levels within the low nanomolar range further offered the possibility to compare effects of Aβ on mitochondrial...
function in a dose-dependent way. As a result, the latter cell model may reflect increased cellular stress evoked by high pathological levels of Aβ, a situation that might be present in familial AD patients (66). Moreover, these two cell models allowed us to study not only effects induced by extracellular Aβ but also the effects of intracellularly generated Aβ and/or other products of the APP-processing pathway, e.g., APP intracellular domain, on mitochondrial function. This is an important advantage over extracellular Aβ treatment, because strong evidence indicates the important role of the intracellular biology of Aβ in the pathogenesis of AD.

Our data suggested that NO plays an important role in Aβ-induced mitochondrial dysfunction and cell death and that intracellularly produced Aβ is specifically relevant. The mitochondrial respiratory chain is sensitive to both NO- and peroxynitrite-mediated damage (30–32). In accordance with these findings, APPsw PC12 cells showed reduced cytochrome c oxidase activity that is probably due to a direct inhibitory effect of NO, whereas the complexes II, III, and V in APPsw cells seemed to be impaired by peroxynitrite. Importantly, APPwt PC12 cells also showed an impairment of cytochrome c oxidase and complex V. Thus, these two complexes are especially susceptible to already low Aβ levels. Recently, it has been shown that APP and Aβ are targeted to mitochondria (63, 68). In accordance, we observed the presence of APP in the mitochondria of APPwt and APPsw PC12 cells. This presence of APP in mitochondria might be involved in the impairment of the mi-
tochondrial respiratory chain. Thus, mitochondrial dysfunction can be induced on the one hand via enhanced NO levels and on the other hand because of a direct effect of Aβ on mitochondria (69), although we can not exclude additional effects of APP itself or other intracellular APP-processing products such as APP intracellular domain on mitochondria.

The reduced cytochrome c oxidase activity in APPsw PC12 cells is consistent with the observation that basal ATP levels of APPsw PC12 cells were significantly reduced compared with control cells. Interestingly, APPwt PC12 cells also showed reduced ATP levels but to a lesser extent than APPsw PC12 cells. We suggest that the very low Aβ levels in APPwt PC12 cells are sufficient to reduce ATP levels but that this depletion of ATP does not induce cell death per se, indicating that APPsw PC12 cells are able to compensate for this deficit under normal conditions. Only after treatment with a secondary insult (in this case oxidative stress), APPsw PC12 cells were no longer able to maintain cellular function and cell death was increased. Afterward, extracellular Aβ treatment showed only weak effects on ATP reduction, suggesting again that Aβ exhibits its effects on mitochondrial function mostly by intracellular effects. In addition, the ATP reduction in APPsw HEK cells was stronger than in APPsw PC12 cells, showing a dose-dependent effect of Aβ on ATP levels. To investigate whether APP or Aβ is mainly responsible for the mitochondrial damage, the intracellular Aβ production was inhibited by the γ-secretase inhibitor DAPT, which is known to reduce intracellular Aβ levels without affecting APP expression (59–61). Our results showed that the DAPT-mediated inhibition of Aβ production lead to a stabilization of mitochondrial function with regard to both a pathogenic hyperpolarization and depolarization of mitochondrial membrane potential in APPsw PC12 cells and APPsw HEK cells, respectively, and to a decrease in NO levels in APPsw PC12 as well as in HEK cells. This finding clearly indicated that increased NO production and mitochondrial dysfunction resulted from increased Aβ levels in APP-transfected PC12 and HEK cells. Our model further strengthened the thesis that Aβ mainly mediates the toxic intracellular effects. However, mitochondrial function did not recover completely to normal levels. This might be due to irreparable damage of the mitochondria by long term expression of Aβ or to APP still persisting in the mitochondrion that may directly interfere with mitochondrial respiratory chain. Very importantly, in 3-month-old APP tg mice, reduced ATP levels can also be observed under basal conditions showing the in vivo relevance of our findings. Of note, the 3 month-old mice exhibited no plaques but already detectable Aβ levels in the brain (39), probably emphasizing again the very important role of intracellular Aβ in ATP reduction. In 12-month-old amyloid plaque-bearing transgenic mice bearing the Swedish APP mutation, reduced cytochrome c oxidase activity and reduced ATP levels were also found (63). Thus, Aβ impairs the energy metabolism of mitochondria in different AD models, possibly as a very early event in the pathogenesis of the disease, as has been shown for AD patients (70).

We could further exclude the possibility that the reduction of ATP levels is due to a reduced number of mitochondria in APPsw PC12 cells, because APPsw transfection rather increased the amount of mitochondria, possibly due to an adaptation process, in response to the increase in NO and reduction of ATP, respectively. This is in accordance with other findings demonstrating a stimulation of the mitochondrial biogenesis by NO (71). Nevertheless, the increased number of mitochondria was not sufficient to compensate the reduced ATP levels in APPsw PC12 cells.

Based on our data and the knowledge regarding the effects of NO on mitochondrial function (30), we proposed a hypothetical sequence of events linking Aβ, NO production, ATP depletion, and mitochondrial membrane potential with caspase pathway and neuronal cell loss.

Under physiological conditions, the mitochondrial membrane potential was estimated at ~150 to ~180 mV with respect to the cytosol. Following acute stimulation with extracellular Aβ, we and other groups (72) found a slight decrease in mitochondrial membrane potential at rather high non-physiological Aβ concentrations. This might have resulted from an inhibition of respiratory chain complexes by increased NO levels leading to a drop in the electron transport and consequently in proton extrusion.

In contrast, APPsw-transfected PC12 cells, which chronically secrete Aβ levels in the picomolar range, showed activation of a defense response in an attempt to maintain cellular function and viability. After an initial decrease of mitochondrial membrane potential, chronic inhibition of the respiratory chain by increased NO levels resulted in hyperpolarization of mitochondrial membrane potential, probably due to an increase in the rate of glycolysis followed by the entering of glycolytic ATP into the mitochondria (73–75). Consistent with this finding, we observed no increased basal apoptosis in APPsw PC12 cells. Interestingly, APPsw HEK cells, which express chronically high Aβ levels, have no possibility to adapt. This is probably due to the prolonged increase in the generation of superoxide anions in the presence of high Aβ levels resulting in the formation of peroxynitrite and thereby facilitating the opening of the permeability transition pore (76, 77). This finally leads to the collapse of mitochondrial potential and the release of mitochondrial proteins including cytochrome c and Smac that in turn activates caspase cascade and cell death. Consistently, we found increased basal apoptotic levels in APPsw HEK cells. A similar situation exists in the brains of APP tg mice at an age of 3 months, which have detectable high Aβ levels in the brain but before the onset of plaque formation. The brain cells of these mice showed a significantly decreased mitochondrial membrane potential. Thus, we have distinct effects of Aβ on mitochondrial function dependent on acute or chronic exposure and on low and high concentration levels of the peptide. These different effects might play a role in sporadic and familial forms of the disease.

However, when APPsw PC12 cells were stressed with an AD-relevant secondary insult, e.g., oxidative stress, the cells were no longer able to compensate for mitochondrial dyshomoeostasis and the mitochondrial membrane potential significantly decreased. In addition, the ATP reduction after oxidative stress was stronger in APPsw PC12 cells than in control cells. Thus, the increased Aβ production at physiological levels enhanced the vulnerability against mitochondrial dysfunction after oxidative stress in APPsw PC12 cells, a process that might be also relevant in sporadic AD. As we could show, a shift in the Bcl-xL/Bax ratio toward Bax might be involved in the higher sensitivity of APPsw cells to mitochondrial failure after oxidative stress. Secondary stress insult finally lead to the release of mitochondrial proteins, e.g., Smac and AIF, which play a role within the cell death cascade (78, 79). The increased Smac release in APPsw cells might explain the higher caspase 9 and caspase 3 activity of APPsw PC12 cells as previously demonstrated (9). In contrast, AIF seems to play only an important role in the late phase of apoptosis in APPsw PC12 cells. Thus, one can speculate that, also in humans, increased Aβ accumulation and associated mitochondrial toxicity are contributing factors in the pathogenesis of AD syndromes. At the beginning, the damaging effects of low physiological concentrations of Aβ could be overcome by an adaptive response. However, when age-related secondary stress occurs, pronounced
mitochondrial impairment might lead to the induction of cell death processes, whereas in familial AD, high Aβ load might be directly responsible for mitochondrial and cellular dysfunction. In summary, we showed novel and distinct actions of Aβ on mitochondria in vitro and in vivo that may contribute to the pathogenic outcome.

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