Mitochondrial amyloid-beta peptide: Pathogenesis or late-phase development?

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Mitochondrial amyloid-beta peptide: Pathogenesis or late-phase development?

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Abstract. Mitochondrial and metabolic dysfunction have been linked to Alzheimer's disease for some time. Key questions regarding this association concern the nature and mechanisms of mitochondrial dysfunction, and whether such changes in metabolic properties are pathogenic or secondary, with respect to neuronal degeneration. In terms of mitochondria and Alzheimer's, altered function could reflect intrinsic properties of this organelle, potentially due to mutations in mitochondrial DNA, or extrinsic changes secondary to signal transduction mechanisms activated in the cytosol. This review presents data relevant to these questions, and considers the implication of recent findings demonstrating the presence of amyloid-β peptide in mitochondria, as well as intra-mitochondrial molecular targets with which it can interact. Regardless of the underlying mechanism(s), it is likely that mitochondrial dysfunction contributes to oxidant stress which is commonly observed in brains of patients with Alzheimer's and transgenic models of Alzheimer's-like pathology.

Keywords: Respiratory chain complex, Alzheimer's disease, neurodegeneration, reactive oxygen species, apoptosis

1. Introduction

Changes in brain energy metabolism are fundamental to the biology of Alzheimer's Disease (AD), suggesting the possibility that amyloid-β peptide (Aβ) impacts on pathways important for metabolic homeostasis. Several key findings link mitochondria to Aβ-induced cellular perturbation. First, intact/functional mitochondria are required for Aβ-induced cytotoxicity [1]. Second, mitochondrial dysfunction, especially at the level of cytochrome c oxidase/complex IV (COX), is a consistent finding in AD-affected tissues, as well as in platelet mitochondria derived from AD patients [2–6]. Third, consistent with metabolic stress, gene expression profiling in a transgenic model of AD (Tg 2576 mice) has shown early upregulation of genes related to mitochondrial energy metabolism in animals as young as 2 months of age [7]. Such mitochondrial dysfunction has the capacity to produce reactive oxygen species (ROS), which can impact the processing of amyloid-β precursor protein (AβPP), intracellular accumulation of Aβ and cellular energetics, such as the level of ATP. Finally, certain mutations in mitochondrial (mt) DNA have been selectively identified in the control region from patients with AD which reduce transcription and are likely to decrease oxidative phosphorylation in the brain [8]. These results suggest a close link between AD and mitochondrial properties. This conclusion is further underscored by recent observations showing that Aβ can accumulate in mitochondria [9] where it engages specific molecular targets such as Aβ binding alcohol deydrogenase (ABAD) and HtrA2/Omi [10,11].

The above observations provide the background for this review, which presents data supporting links between mitochondrial dysfunction and the pathogenesis/progression of AD.

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2. Mitochondrial dysfunction in AD

Mitochondria contribute to neuronal homeostasis on many levels, including maintenance of the cellular energy charge, intracellular calcium levels [12], regulation of programmed cell death [13] and limiting oxidant stress. Electrons continuously flow through the electron transport chain, as reducing equivalents extracted from nutrients generate the proton motive force essential for aerobic generation of ATP. Morphological alterations in mitochondria have been noted in AD brain, though these changes are limited to vulnerable neurons in the hippocampus and neocortex [14,15]. Labeling of neurons by in situ hybridization with oligonucleotide probes for mtDNA demonstrated an increased signal in AD, but this was apparently due to accumulation of mitochondrial markers in the cytoplasm associated with autophagosomal degradation of mitochondria [14]. In vulnerable portions of AD brain, morphometry showed a decrease in the area of intact mitochondria. Other investigators [15] have noted morphologic changes in AD mitochondria, including changes in cristae, accumulated material and decreased size, compared with controls.

Even prior to the above alterations in mitochondrial morphology, investigators have suspected links between dysfunctional mitochondrial energetics/electron transport chain and AD [16]. Several lines of evidence have provided support for this hypothesis.

1) Imaging studies using positron emission tomography (PET) have shown hypometabolism, especially in the temporoparietal cortex of patients with AD [17–21] at an early clinical stage of the disease.
2) Experiments have also been performed using the cytoplasmic hybrid (or cybrid) technique [22] in which mitochondrial DNA/mitochondria are introduced into a host cell which has been depleted of mitochondria. Platelet mitochondria, from AD patients or age-matched controls, have been transferred into neuronal-like replicating (neuroblastoma; SH-SY5Y) host cells devoid of mitochondria. These studies have provided insights into a link between a defect in the electron transport chain, oxidant stress and generation of Aβ. Cybrids made with AD mitochondria displayed decreased cytochrome oxidase activity and increased evidence of reactive oxygen species (ROS). In parallel, such decreased mitochondrial function was associated with increased secretion of Aβ (using host neuronal-related cells and platelets from AD patients) [23,24], and vulnerability to the effects of Aβ, including an exaggerated decrease in the mitochondrial membrane potential, release of cytochrome c and activated caspase c resulting in cell death [24]. These data suggest that mitochondrial DNA contains information leading to decreased cytochrome c oxidase activity which could be a fundamental defect resulting in a cycle of increasing oxidant stress and generation of Aβ (see below). The observation that nitric oxide (NO) can be induced in settings of cell stress, and that NO also suppresses cytochrome c oxidase activity, provides another mechanism reinforcing this pathway [25,26].
3) The activity of mitochondrial enzymes involved in the Krebs cycle and electron transfer chain components has also been analyzed in AD. Earlier studies on autoptic human brain from AD patients have demonstrated decreases in pyruvate dehydrogenase complex activity [27], as well as α-ketoglutarate dehydrogenase [28,29]. However, the most consistent reported abnormality in mitochondrial enzyme activity is in cytochrome c oxidase [2–6,30].

3. Oxidant stress in AD

AD is closely associated with oxidant stress on many levels. As the principal source of reactive oxygen species (ROS) in cells, mitochondria are a key potential generator of oxidants. In addition, membrane-associated NADPH oxidase, whose activation can be triggered following engagement of cell surface receptors [31–34] by Aβ, has the potential to produce ROS. Mitochondrial DNA is especially susceptible to oxidative damage in view of the limited repair mechanisms, lack of histones and physical contiguity to the source of ROS [35,36]. For example, 8-hydroxy-2'-deoxyguanosine, an oxidized base, has proven to be a useful marker of oxidant stress [37]. Levels of several oxidized bases in AD brain were found to be higher in frontal, parietal and temporal lobes compared to age-matched controls [38]. MtDNA displayed about 10-fold greater levels of oxidized bases than nuclear DNA. In addition, protein and lipid oxidation has been noted in AD [39,40]. Proteins and lipids damaged by oxidant stress, which have been demonstrated in AD, may display altered function, and products of lipid oxidation, such as the aldehyde 4-hydroxy-2-nonenal, can have toxic effects. Oxidant stress is also manifested in

AD-affected tissue as displayed by activation of NF-κB and induction of genes such as heme oxygenase type 1 [41–43], though multiple pathways can trigger these mechanisms.

Another level at which oxidant stress may be relevant to the pathogenesis of AD involves generation of Aβ and induction of BACE-1 [44,45]. Studies in Down's syndrome brains/neurons have shown that cortical neurons from fetal Down's Syndrome undergo programmed cell death in culture, compared with controls. Oxygen-free radical scavengers had the capacity to prevent such apoptosis [46]. Further study of astrocyte and neuronal cultures from fetal Down's syndrome brain displayed changes in processing of AβPP which resulted in intracellular accumulation of Aβ42. Inhibition of mitochondrial function using an uncoupling agent (carbonyl cyanide m-chlorophenylhydrazone, CCCP) in astrocyte cultures reproduced changes in AβPP processing observed in Down's Syndrome brain [47]. There are additional links between intracellular production of Aβ and oxidant stress. In studies with NT2 cells, incubation with 4-hydroxy-2,3-nonenal resulted in elevated levels of intracellular Aβ [48]. These data, though fragmentary and principally in cell culture, suggest linkage between generation of intracellular Aβ and oxidant stress.

Generation of oxidants in response to Aβ can occur by multiple mechanisms. One mechanism particularly relevant in the setting of extracellular Aβ is engagement of cellular receptors. Aβ binding to RAGE results in the generation of ROS due, at least initially, to activation of NADPH oxidase [31,49]. At later times, probably, ROS are produced by mitochondrial-dependent pathways as well [50]. This mechanism may be especially relevant in astrocytes/microglia, which display NADPH oxidase-like enzymatic activity [32]. These data suggest pathways by which ROS in astrocytes/microglia might directly and/or indirectly (via mediators/cytokines) impact on neurons.

Mitochondrial dysfunction is an important source of ROS. When there is a distal blockade in mitochondrial function, as at complex IV, then there is a tendency for electrons to leak to acceptors more proximally and to generate ROS, rather than water. This is particularly relevant in AD where the most consistent defect in the electron transport chain is at complex IV [2–6].

4. Amyloid Precursor Protein (AβPP) and γ-secretase are present in mitochondria

Recent studies have demonstrated that both AβPP and γ-secretase are present in mitochondria, suggesting the possibility that Aβ might be generated in this cellular compartment. Anandatheerthavarada and colleagues [51] have shown that AβPP has an amino-terminal signal that targets it to mitochondria, in addition to endoplasmic reticulum (this finding has only been shown in this paper). This was similar to what these authors noted previously with P450A1, P4502B1 and P4502E1 proteins, based on the similarity of amino terminal chimeric signals [52]. In particular, positively charged residues 40, 44 and 51 in AβPP were essential for mitochondrial targeting. The orientation of AβPP in mitochondria, based on cross-linking studies, seemed to be with the molecule's amino terminus in a transmembrane orientation at the inner mitochondrial membrane and in contact with mitochondrial translocase proteins. The C-terminus (about 73 kDa) was facing the cytosol. Because of the presence of the acidic domain of AβPP (residues 220–290), the molecule is subject to translocation arrest, whereby AβPP becomes trapped in the mitochondrial membrane due to incomplete translocation. Thus, AβPP accumulates in the mitochondrial membrane, and this appears to have consequences for mitochondrial function, including decreased cytochrome c oxidase activity, diminished levels of cellular ATP and decreased mitochondrial transmembrane potential, based on *in vitro* studies.

As a counterpart to these studies on AβPP in mitochondria, recent experiments have shown the presence of γ-secretase in mitochondria. The γ-secretase complex consists of the catalytic core, due to presenilins 1 or 2, as well as three other proteins; nicastrin, APH-1 (anterior pharynx-defective phenotype) and PEN-2 (PS-enhancer) [53]. Functional complexes containing presenilin have been demonstrated in multiple intracellular compartments, including endolysosomes, the plasma membrane, ER-Golgi boundary, trans-Golgi network, lipid rafts of post-Golgi/endosome membranes, and mitochondria [54–57]. In the context of mitochondria, γ-secretase activity appears to be associated with the mitochondrial inner membrane where immunolocalization studies have identified presenilin 1 and nicastrin [57]. It should be noted that in view of the orientation of AβPP in mitochondria, with the C-terminus exposed to the cytoplasm, it would be difficult for γ-secretase at the inner membrane to appropriately interact with its cleavage sites. Furthermore, the functional significance of such secretase activity is not at all clear at the present time.

Furthermore, in order for AβPP to be a substrate for γ-secretase, it is first cleaved by BACE-1 (β-site AβPP cleaving enzyme). It would appear that endosomes and

plasma membrane are the principal sites of BACE-1 cleavage of AβPP [58]. No BACE-1 activity has been detected in mitochondria. Thus, based on the lack of BACE-1 in mitochondria and the orientation of AβPP in mitochondrial membranes, it appears unlikely that Aβ is generated in mitochondria.

5. Aβ is present in mitochondria

Although Aβ was initially thought to be generated principally at the cell membrane and released extracellularly, the existence of intracellular Aβ is now well-established [59–69]. Sites of intracellular Aβ generation include the endoplasmic reticulum/Golgi compartment as well as endolysosomes [70–75], and intracellular accumulation occurs in these same portions of the cell as well as in multivesicular bodies [61,64,76]. Because of links between mitochondrial dysfunction and AD, we considered the possibility that Aβ might have a direct effect on mitochondria, potentially by entering the organelle. This led us to perform experiments in transgenic mice (Tg) overexpressing mutant AβPP [77] and AD brain patient samples [9]. In order to ensure mitochondrial integrity, postmortem AD samples and nondemented control brains were harvested with a time delay of less than 3.5 hours. Biochemical studies on mitochondria purified from cerebral cortices of Tg mAPP mice and AD brains demonstrated the presence of Aβ in highly purified fractions. Importantly, protease treatment of mitochondria, to release Aβ adsorbed to the organelle's surface, did not remove immunoreactive Aβ, indicating the presence of amyloid-β peptide in a membrane enclosed compartment.

Morphologic studies were also performed to assess the possible localization of Aβ to mitochondria [9]. Using confocal microscopy, Aβ42 was shown to colocalize with HSP60, the latter a marker of mitochondrial matrix. Neurons in the cortex (Fig. 1A) and hippocampus (Fig. 1B) displayed an overlapping distribution of Aβ42 and HSP60 antigens. About 40% and 20% of the area occupied by mitochondria could be colocalized with Aβ in the cerebral cortex and hippocampus, respectively. In contrast, nonTg littermates showed undetectable Aβ42 antigen (Fig. 1C). When comparable studies were performed in AD brain, colocalization of Aβ was also observed in mitochondria (about 40% and 70% of the area occupied by mitochondria costained with antibody to Aβ in temporal lobe and hippocampus, respectively). In human nondemented control samples, accumulation of Aβ was seen, but to a much lesser extent than in AD.

The time course of accumulation of Aβ in mitochondria in brains of Tg AβPP mice was assessed in animals from 4–24 months of age [9]. The most rapid phase of Aβ accumulation appeared to be from 8–12 months of age, for both Aβ42 and Aβ40 (Fig. 2A–B). However, levels of Aβ42 were considerably higher than Aβ40, as is true for intracellular Aβ in other compartments. Mitochondrial Aβ was first detectable at 4 months of age, before significant extracellular deposition of Aβ in this animal model. After 12 months of age, there was only a slight increase in mitochondrial Aβ at higher ages.

It was essential to determine if the association of Aβ with mitochondria could be correlated with functional perturbations of the organelle [9]. Analysis of mitochondria from Tg AβPP mice demonstrated that by 8 months of age, there was a trend towards lower levels of the respiratory control ratio, compared with nonTg controls. Furthermore, analysis of respiratory complexes showed reduced complex III and complex IV activity by 12 months of age in Tg AβPP mitochondria, versus controls (non-Tg littermates). Although these data represent correlations, rather than cause-effect relationships, it appears that the presence of mitochondrial Aβ is associated with reduced activity of certain key enzymes in the respiratory chain.

It is important to note that these results raise multiple questions regarding Aβ and mitochondria: how does Aβ enter mitochondria? Which mitochondrial compartments does Aβ reside in? Does Aβ impact on mitochondrial function? If so, what mechanisms are involved? Does mitochondrial Aβ affect cellular properties, such as susceptibility to apoptosis, regulation of cytosolic calcium or respiratory chain function?

6. Targets of Aβ in mitochondria

The presence of Aβ in mitochondria, taken together with impaired mitochondrial function, suggests that amyloid targets vulnerable mitochondrial structures thereby impacting on overall properties of this organelle. In view of the known effect of Aβ to destabilize membranes, especially at higher concentrations, it is certainly possible that nonspecific mechanisms are responsible for Aβ-induced mitochondrial dysfunction [78–80]. Pores in the inner mitochondrial membrane would have the capacity to dissipate the proton motive force which drives ATP production. Another

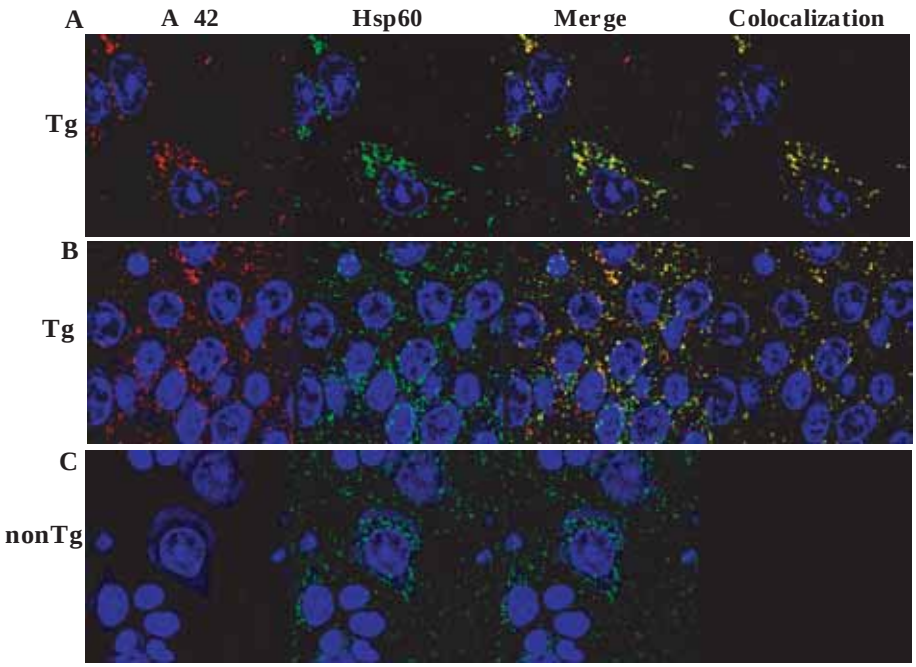

Fig. 1. Confocal images from transgenic mice overexpressing mutant AβPP stained for Aβ42 (red) and Hsp60 (green) in the cerebral cortex (A,C) and hippocampus (B) at 10 months of age (A,B) and nontransgenic littermates (C). Magnification: x 400. Adapted from reference [9].

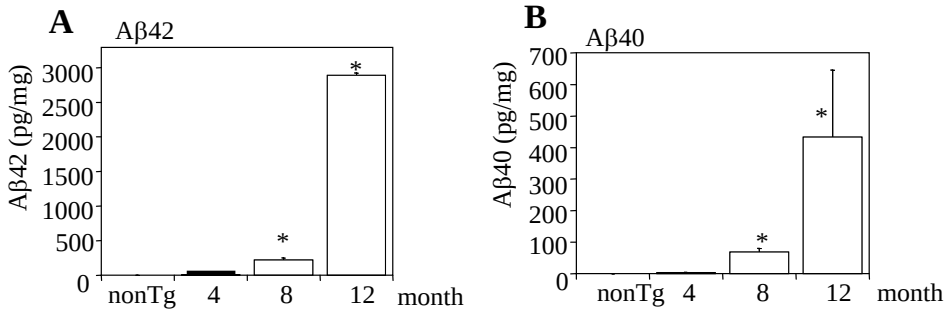

Fig. 2. Accumulation of Aβ in mitochondria from transgenic mice overexpressing mutant amyloid precursor protein. ELISA for Aβ42 (A) and Aβ40 (B) was performed on isolated brain mitochondria from Tg mice of the indicated ages. Adapted from reference [9].

view (complementary or alternative) is that Aβ targets particular molecules within mitochondria by binding to them and altering their function. The appeal of this viewpoint is, depending on the affinity of Aβ for the target, relatively low concentrations of Aβ could modulate function of key components of the mitochondrial regulatory machinery. Such a mechanism could have a global effect on mitochondrial function.

Using the yeast two-hybrid system, our group identified ABAD as a short-chain oxidoreductase which interacted with Aβ and is present in the mitochondrial matrix compartment (as well as other intracellular compartments, such as endoplasmic reticulum) [10,81]. ABAD is an enzyme involved in metabolic homeostasis, particularly in the context of isoleucine degradation [82–85]. The enzyme methyl-3-hydroxybutryryl-CoA dehydrogenase (MHBD), which catabolizes isoleucine and branched-chain fatty acids, is identical to ABAD. Patients deficient in MHBD/ABAD display a severe neurologic syndrome, including progressive neurodegeneration at a young age [86–88]. Although the cause of the toxicity is unknown, it is certainly possible that catabolites in the isoleucine degradation pathway, such as tiglic acid, accumulate and exert untoward effects. *Drosophila* functionally deficient in ABAD (*scully* is the *Drosophila* counter-

part of ABAD) also have a severe phenotype, namely developmental lethality, with multiple abnormalities some of which are reminiscent of defects in fatty acid oxidation [89]. In this context, ABAD participates in fatty acid β-oxidation, as well as the metabolism of β-hydroxybutyrate [82–84]. ABAD shares many features with other members of the family of short-chain dehydrogenase reductases, including binding of an NAD/NADP cofactor and properties of the catalytic site. Its unique properties include participation in isoleucine degradation, presence in mitochondria (as well as, to a lesser extent, endoplasmic reticulum in certain cells), a broad range of substrates, and the ability to bind Aβ and to facilitate Aβ-induced cell stress [81–89].

In the context of metabolic homeostasis, overexpression of ABAD appears to result in a protective phenotype [82]. Transgenic mice with targeted overexpression of ABAD in neurons, using the PDGF B-chain promoter, were more resistant to ischemic stress. Using the transient middle cerebral artery model of stroke, transgenic ABAD mice displayed strokes with reduced infarct volumes and decreased neurologic deficits. To our surprise, ABAD transgenic mice had higher baseline levels of brain ATP, and demonstrated increased flux of β-hydroxybutyrate through the tricarboxylic acid cycle, compared with wild-type animals, under both basal and ischemic conditions. The protective effects of ABAD in the setting of ischemia could be related to its metabolic properties (generation of ATP and enhanced utilization of β-hydroxybutyrate), its direct effect on mitochondrial properties, or other yet-to-be discovered mechanisms.

In view of the presence of Aβ and ABAD in mitochondria, as well as the functional importance of ABAD in homeostasis, it was important to determine if these two molecules formed a complex in mitochondria. If so, we wanted to assess whether ABAD-Aβ complex modified the enzymatic properties of ABAD. Immunoprecipitation studies of AD brain demonstrated complex formation between ABAD and Aβ in brain and mitochondrial extracts [10]. This was consistent with the results of binding studies in which Aβ(1–42), Aβ(1–40) and Aβ(1–20) demonstrated dose-dependent saturable binding to ABAD. The C-terminal portion of Aβ(25–35) did not bind to ABAD, indicating that ABAD-Aβ interaction was not due to nonspecific interaction with aggregated or fibrillar material. This observation also suggested the possibility that Aβ might anchor, via its N-terminus, in ABAD thereby leaving the C-terminus free and allowing it to multimerize with additional Aβ. At the level of morphology, both confocal microscopy and immunoelectron microscopy confirmed colocalization of ABAD and Aβ in AD brain. Such morphologic results were relevant, as the biochemical studies clearly reflected observations in disrupted tissue.

The likelihood that ABAD and Aβ interacted in brain underscored the relevance of determining if there might be a functional implication of this interaction. First, we performed studies to attempt to solve the structure of ABAD-Aβ complex. High resolution crystals were obtained which demonstrated considerable distortion of ABAD structure in the presence of Aβ. Specifically, the NAD cofactor was not bound to ABAD and there were multiple structural changes in the loops of ABAD (L_D, L_E, L_F) whose orientation was important for ABAD activity. Particularly relevant in this regard was the observation that the LD loop had disordered structure in ABAD-Aβ crystals. Comparison of the LD loop of ABAD with other enzymes in this family showed an insertion of eleven amino acids, suggesting the possibility that this might be a site mediating the binding to Aβ. To test this hypothesis, peptides were made spanning this region. These studies indicated that residues 91–119 (including the insertion within the LD loop) were critical for Aβ binding to ABAD.

The relevance of ABAD for promoting Aβ-induced cell stress was demonstrated in studies with transgenic mice overexpressing AβPP and ABAD in neurons [10]. These double transgenic animals displayed increased generation of reactive oxygen species in the brain, by electron paramagnetic resonance spectroscopy, and accelerated impairment of spatial learning/memory (Fig. 3A–B). Cultured neurons from these double transgenic mice showed spontaneous release of reactive oxygen species (the defect resulting in oxygen free radical production appears to occur at complex III) [90] and cytochrome c from mitochondria [10,90]. In fact, neurons cultured from these animals displayed spontaneous apoptosis. Although the precise mechanisms through which ABAD perturbs mitochondrial functions remain to be determined, it is clear that in model systems ABAD-Aβ interaction modulates properties of ABAD. Using purified proteins, addition of Aβ to ABAD decreased the enzyme's activity to a broad range of substrates. *In vivo*, a much more complex set of interactions appears to occur that stimulates formation of reactive oxygen species and other events.

In addition to ABAD, Aβ is likely to interact with components of the mitochondrial import machinery (assuming that Aβ is produced outside mitochondria

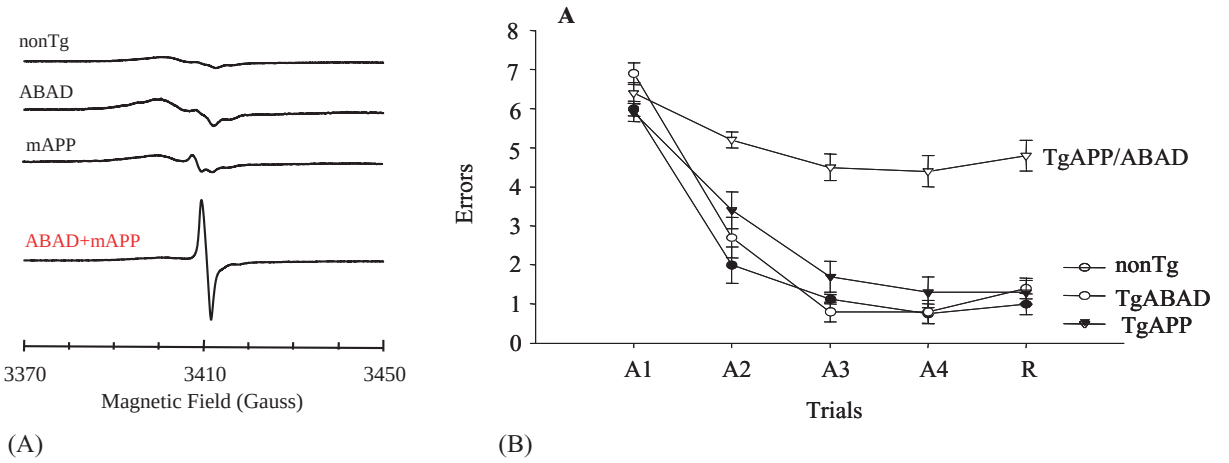

Fig. 3. Panel A. Generation (by EPR spectroscopy) of free radicals in brains of double transgenic mice overexpressing mutant amyloid precursor protein and ABAD under control of the PDGF B chain promoter. Note that the amplitude of the spectra for Tg mutant(m)AβPP, Tg ABAD and non-Tg animals has been increased ten-fold to display spectra which showed only low-level changes compared with the double transgenics (mAβPP/ABAD). Panel B displays spatial learning in 4.5–5 month-old double transgenic mice (compared with other groups) tested in the radial-arm water maze. Adapted from reference [10].

and imported into the organelle; see above) and mitochondrial chaperones. In the latter case, Aβ has been shown to bind HSP70 and HSP16 family members, based on immunoprecipitation assays in a transgenic *C. elegans* system [91]. Another apparently specific Aβ-interacting protein is HtrA2/Omi [11], a serine protease with proapoptotic properties [92–96]. It serves as a binding protein for inhibitor of apoptosis protein (IAP). HtrA2/Omi is imported into mitochondria via a mitochondrial targeting sequence which is subsequently autocatalytically removed after entering the organelle. Apoptotic stimuli cause release of HtrA2/Omi from mitochondria, which then binds to IAPs [92–96]. Although the functional significance of the interaction of Aβ with HtrA2/Omi is unclear, in terms of its impact on programmed cell death, it is possible that modulation of HtrA2/Omi activity occurs.

7. Hypothesis

The observations cited in this review highlight a relationship between altered mitochondrial function, Aβ and Alzheimer's disease. While it is not possible to draw conclusions as to which comes first and whether mitochondrial perturbation is pathogenic in AD, it is clear that mitochondrial dysfunction is associated with AD. We propose that impaired mitochondrial function, at the level of complexes III and/or IV, promotes leakage of electrons resulting in generation of reactive oxygen species. Such free radicals perturb cellular and mitochondrial properties further deranging neuronal/microglial properties. However, there are many possibilities for mechanisms underlying such mitochondrial dysfunction. For example, the interaction of extracellular Aβ with membrane receptors, such as RAGE, could trigger activation of NADPH oxidase resulting in free radical formation. Alternatively, or in addition, intrinsic defects in complex IV (nitric oxide also decreases function of complex IV) would tend to cause accumulation of reduced components of the electron transport chain, promoting the possibility of electron leakage. Mutations in mitochondrial DNA, especially those in the control region, which would impact on transcription of genes essential for mitochondrial function could also affect the integrity of cellular respiration. The presence of Aβ within mitochondria provides another level of complexity in this setting, as mitochondrial Aβ could seek out particular molecular targets, such as ABAD or HtrA2/Omi. Alternatively, changes in mitochondrial properties in AD could be indirect, potentially due to increased generation of nitric oxide and other changes related to signal transduction mechanisms in the cytosol. Further analysis of mitochondrial properties in AD will be necessary to dissect this situation and understand if mitochondrial perturbation is pathogenic in this neurodegenerative disease.

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