Journal of Neurochemistry, 2006, **96**, 1–13 doi:10.1111/j.1471-4159.2005.03530.x

REVIEW

Amyloid precursor protein-mediated free radicals and oxidative damage: Implications for the development and progression of Alzheimer's disease

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Abstract

Alzheimer's disease (AD) is a late-onset dementia that is characterized by the loss of memory and an impairment of multiple cognitive functions. Advancements in molecular, cellular, and animal model studies have revealed that the formation of amyloid beta (Aβ) and other derivatives of the amyloid precursor protein (APP) are key factors in cellular changes in the AD brain, including the generation of free radicals, oxidative damage, and inflammation. Recent molecular, cellular, and gene expression studies have revealed that Aβ enters mitochondria, induces the generation of free radicals, and leads to oxidative damage in post-mortem brain neurons from AD patients and in brain neurons from cell models and transgenic mouse models of AD. In the last three decades, tremendous progress has been made in mitochondrial research and has provided significant findings to link mitochondrial oxidative damage and neurodegenerative diseases such as AD. Researchers in the AD field are beginning to recognize the possible involvement of a mutant APP and its derivatives in causing mitochondrial oxidative damage in AD. This article summarizes the latest research findings on the generation of free radicals in mitochondria and provides a possible model that links Aβ proteins, the generation of free radicals, and oxidative damage in AD development and progression.

Keywords: *in vitro* studies, mitochondria, mitochondrial gene expression, oxidative damage, reactive oxygen species, transgenic mice.

J. Neurochem. (2006) **96**, 1–13.

Alzheimer's disease (AD) is a late-onset, progressive, age-dependent neurodegenerative disorder, characterized clinically by the impairment of cognitive functions and changes in behavior and personality (Selkoe 2001; Mattson 2004; Reddy and McWeeney 2005; Tanzi and Bertram 2005). AD is associated with the presence of intracellular neurofibrillary tangles and extracellular amyloid beta (Aβ) plaques, a loss of neuronal subpopulations, synaptophysin immunoreactivity of presynaptic terminals, cholinergic fibers, the proliferation of reactive astrocytes, and microglia oxidative damage (Selkoe 2001; Reddy and Beal 2005; Reddy and McWeeney 2005; Tanzi and Bertram 2005).

Although AD occurs in both familial and sporadic forms, genetic mutations are responsible for causing AD in only 2% of AD cases, but the causal factor(s) for the vast majority of AD cases is still unknown. Recently, however, several reports and reviews have suggested that mitochondrial abnormalities and oxidative stress play a role in sporadic AD (Hirai *et al*. 2001; Cash *et al*. 2002; Swerdlow and Kish 2002; Coskun *et al*. 2004; Keil *et al*. 2004; Manczak *et al*. 2004; Swerdlow and Khan 2004; Behl 2005; Reddy and Beal

Received August 16, 2005; revised manuscript received September 12, 2005; accepted September 12, 2005.

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Abbreviations used: Aβ, amyloid beta; ABAD, Aβ-binding alcohol dehydrogenase; AD, Alzheimer's disease; APP, amyloid precursor protein; ATP, adenosine triphosphate; BACE, beta-secretase; ETC, electron transport chain; H_2O_2, hydrogen peroxide; HNE, 4-hydroxynonenal; mtRNA, mitochondrial RNA; mtDNA, mitochondrial DNA; NO, nitric oxide; $O_2^{\bullet -}$, superoxide anion; $^{\bullet}OH$, hydroxyl radical; ROS, reactive oxygen species; TCA, tricarboxylic acid.

2005; Zhu *et al*. 2005). This study reviews research that suggests a connection between derivatives of the mutant amyloid precursor protein (APP) and the generation of free radicals, and mitochondrial oxidative damage in the development and progression of AD.

Formation of reactive oxygen species in the mitochondria

Reactive oxygen species (ROS) is a term used to describe free radicals derived from molecular oxygen. Molecular oxygen in the ground state is a bi-radical, containing two unpaired electrons in the outer shell (Turrens 2003; Cadenas and Davies 2000). Since the two unpaired electrons have the same spin and since oxygen can only react with one electron at a time, oxygen is not very reactive with the two electrons in a chemical bond. On the other hand, if one of the two unpaired electrons is excited and changes its spin, the resulting species (known as a singlet oxygen) becomes a powerful oxidant, as the two electrons with opposing spins can quickly react with other pairs of electrons (Turrens 2003). The reduction of oxygen by one electron at a time produces relatively stable intermediates. Superoxide anion ($O_2^{\bullet -}$), the product of oxygen that is reduced by one electron, is the precursor of most ROS and is a mediator in oxidative chain reactions. Dismutation of $O_2^{\bullet -}$, either spontaneously or through a catalytic reaction by superoxide dismutases, produces hydrogen peroxide (H_2O_2), which in turn may be fully reduced to water or partially reduced to a hydroxyl radical ($^{\bullet}OH$), one of the strongest oxidants in nature (Turrens 2003). The formation of $^{\bullet}OH$ is catalyzed by reduced-transition metals, such as copper and iron, that, in turn, may be re-reduced by $O_2^{\bullet -}$ and may propagate the entire ROS process (Liochev and Fridovich 1999). In addition, $O_2^{\bullet -}$ may react with other radicals, including nitric oxide (NO) in a reaction controlled by the rate that both radicals diffuse. The resulting product, peroxynitrite, is also a very powerful oxidant (Beckman and Koppenol 1996; Radi *et al*. 2002). The oxidants derived from NO have been recently called reactive nitrogen species.

The production of mitochondrial $O_2^{\bullet -}$ occurs primarily at discrete points in the electron transport chain (ETC) at complexes 1 and 3, and in components of tricarboxylic acid (TCA), including α-ketoglutarate dehydrogenase (Finkel and Holbrook 2000; Starkov *et al*. 2004) (see Fig. 1). When the ETC is inhibited, the electrons accumulate in the early stages of the ETC (complex 1 and coenzyme Q), where they are donated directly to molecular oxygen, to give an $O_2^{\bullet -}$ (Wallace 1999). The $O_2^{\bullet -}$ is detoxified by the mitochondrial Mn-superoxide dismutase to give H_2O_2. Then H_2O_2 is converted into H_2O by either glutathione peroxidase or catalase. Chronic exposure to ROS can result in oxidative damage to mitochondrial and cellular proteins, lipids, and nucleic acids, and acute exposure to ROS can inactivate theTCA-cycle aconitase and the iron-sulfur centers of ETC at complexes 1, 2, and 3, resulting in a shutdown of mitochondrial energy production (Wallace 1999; Reddy and Beal 2005).

Another important aspect of ETC is the generation of ROS, which is a physiologically important by-product of respiration (Reddy and Beal 2005). During the transfer of electrons to molecular oxygen, an estimated 1% to 5% of electrons in the respiratory chain lose their way and participate in forming $O_2^{\bullet -}$. Molecular oxygen generated from this loss of electrons ultimately activates the mitochondrial permeability transition pore and destroys the cell apoptosis. In contrast with other oxidant-producing systems of the cell, mitochondria are required for the production of ATP and are present in relatively high numbers in essentially all cells of the body (Reddy and Beal 2005).

Free-radical generation by the inner mitochondrial membrane and matrix

Figure 1 illustrates the generation of free radicals in the mitochondria. As shown in Fig. 1, respiratory complexes 1 and 3 leak electrons to oxygen, which produces primarily superoxide radicals (or $O_2^{\bullet -}$). $O_2^{\bullet -}$ anions are dismutated by manganese superoxide dismutase and generate H_2O_2 and oxygen. H_2O_2 is then converted to H_2O and oxygen by means of either catalase or glutathione peroxidase. Complex 1 generates $O_2^{\bullet -}$ only in the matrix of mitochondria, in contrast to complexes 2, 4, and 5, which seem to play no role in $O_2^{\bullet -}$ generation in the ETC. Complex 3, on the other hand, generates $O_2^{\bullet -}$ toward both the intermembrane space and the matrix (Muller *et al*. 2004). Recent biochemical studies suggest that components of a TCA cycle, including α-ketoglutarate dehydrogenase, also generate $O_2^{\bullet -}$ in the mitochondrial matrix (Starkov *et al*. 2004). Increased steady-state concentrations of $O_2^{\bullet -}$ may reduce transition metals, such as iron and copper, which in turn may react with H_2O_2 to produce $^{\bullet}OH$, or the increased $O_2^{\bullet -}$ may react with NO to form peroxynitrite. Both $^{\bullet}OH$ and peroxynitrite are strong oxidants that indiscriminately react with nucleic acids, lipids, and proteins (Turrens 2003; Cadenas and Davies 2000).

Generation of free radicals by the outer mitochondrial membrane

Flavoprotein (monoamine oxidase), localized on the outer mitochondrial membrane, catalyzes the oxidative deamination of primary aromatic amines. This deamination is a quantitatively large source of H_2O_2 that contributes to an increase in the steady-state concentrations of ROS within both the mitochondrial matrix and the cytosol (Han *et al*. 2003). H_2O_2, produced during the oxidative deamination of catecholamines, has been identified as likely involved in neurodegenerative disorders, such as AD and Parkinson's

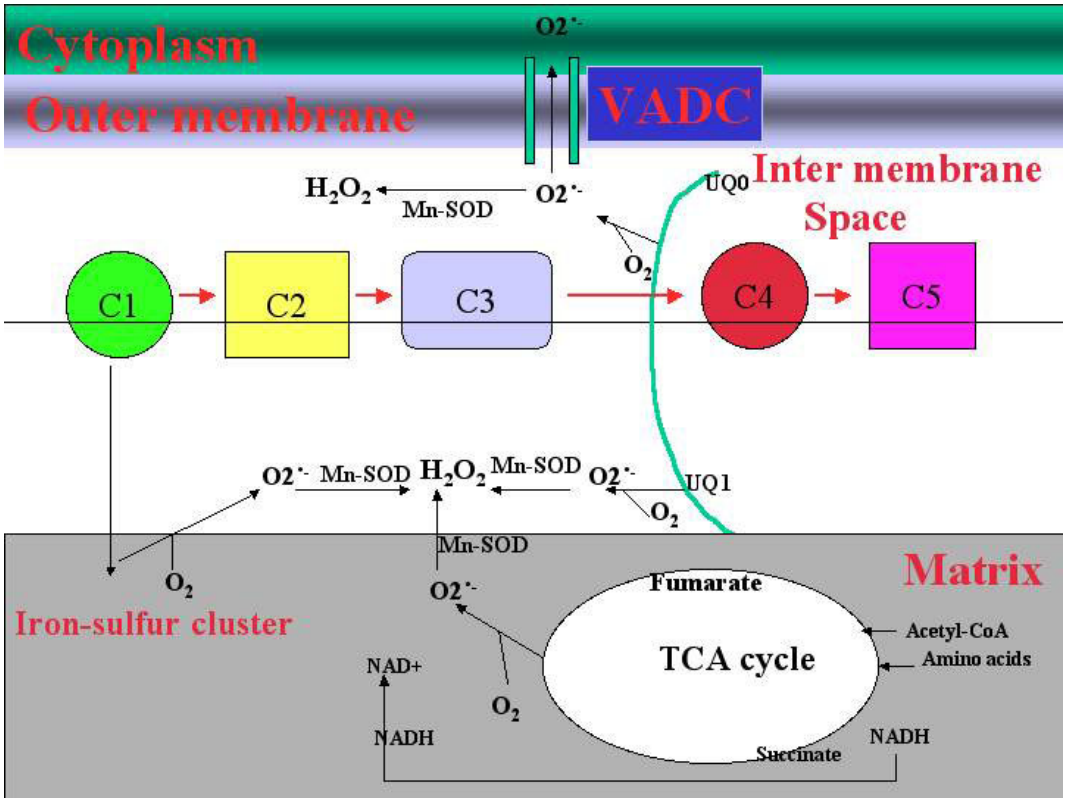

Fig. 1 Sites of free radical generation in the mitochondria. In the respiratory chain, complexes 1 and 3 (C1 and C3) leak electrons to oxygen, producing primarily superoxide radicals (or $O_2^{\bullet-}$). The $O_2^{\bullet-}$ are dismutated by manganese superoxide dismutase (Mn-SOD) to generate H_2O_2 and oxygen. Complex 1 (C1) generates $O_2^{\bullet-}$ only toward the matrix, whereas in the electron transport chain, complexes 2 (C2), 4 (C4), and 5 (C5) seem to play no role in $O_2^{\bullet-}$ generation. Complex 3 (C3), on the other hand, generates $O_2^{\bullet-}$ toward both the intermembrane space and the matrix. In addition, the components of tricarboxylic acid (TCA), including α-ketoglutarate dehydrogenase, also generate $O_2^{\bullet-}$ in the matrix.

disease, presumably via oxidative damage to the mitochondrial membrane (Cohen 1983; Fhan and Cohen 1992).

In vivo, $O_2^{\bullet-}$ is produced both enzymatically and nonenzymatically. Enzymatic sources include NADPH oxidases, which are located on the cell membranes of polymorphonuclear cells, macrophages, and endothelial cells (Babior 2000; Babior *et al*. 2002; Vignais 2002). The proteolytic conversion of xanthine dehydrogenase to xanthine oxidase provides another enzymatic source for both $O_2^{\bullet-}$ and H_2O_2 (and therefore constitutes a source of $^{\bullet}OH$) and has been proposed to mediate deleterious processes *in vivo* (Yokoyama *et al*. 1990). The nonenzymatic production of $O_2^{\bullet-}$ occurs when a single electron is directly transferred to oxygen by reduced coenzymes or to prosthetic groups (for example, flavins or clusters of iron sulfur) by xenobiotics that certain enzymes have previously reduced. The mitochondrial ETC contains several redox centers that may leak electrons to oxygen, constituting the primary source of $O_2^{\bullet-}$ in most tissues.

Although several laboratories in the last three decades have identified a variety of mitochondrial sources of $O_2^{\bullet-}$, the physiological link between disease progression in neurodegenerative diseases, such as AD, and free radicals is still not clear. Superoxide formation occurs on the outer mitochondrial membrane, in the matrix, and on both sides of the intermitochondrial membrane (Fig. 1). The $O_2^{\bullet-}$ that is generated in the matrix is eliminated in that compartment, whereas the $O_2^{\bullet-}$ of the intermembrane space may be carried to the cytoplasm via voltage-dependent anion channels (see Fig. 1) (Han *et al*. 2003).

Generation of amyloid beta and free radicals

In the brains of AD patients, Aβ plaques or deposits have been found to result from a cleaved APP of 4 kDa, which is a 39–42 amino acid peptide (Selkoe 2001). This cleavage occurs due to proteases – referred to as β and γ secretase on either side of the Aβ sequence of APP molecule – and results in the production of the full Aβ peptide (Haass *et al*. 1992;

Shoji *et al*. 1992). Aβ, the primary constituent of Aβ plaques in AD, is hypothesized to cause neuronal damage and cognitive failure via the generation of free radicals, mitochondrial oxidative damage, synaptic failure and inflammatory changes in the brains of AD patients (Selkoe 2001; Mattson 2004; Reddy and Beal 2005; Reddy and McWeeney 2005; Tanzi and Bertram 2005). However, the precise mechanisms of AD progression are still not clearly understood.

To determine the precise connection between Aβ deposits and the generation of free radicals, using multiphoton imaging, McLellan *et al*. (2003) recently showed a direct association between Aβ deposits and free-radical generation *in vivo* in live, transgenic mouse models of AD and in analogous *ex vivo* experiments using postmortem brain tissue from AD patients. Before imaging the neurons with a near infrared laser, they applied two fluorogenic compounds that become fluorescent only after oxidation. They observed fluorescence associated with dense core plaques, but not with diffuse plaques, as determined by the subsequent addition of thioflavine S and immunohistochemistry of the Aβ plaques. Systemic administration of *N-tert*-butyl-phenylnitrone, a free-radical spin trap, greatly reduced the oxidation of the fluorescent. These data showed that a subset of Aβ plaques produces free radicals in living mouse models of AD and in human AD tissue. Antioxidant therapy has been found to neutralize these highly reactive molecules (McLellan *et al*. 2003) and may therefore be of therapeutic value in treating patients with AD.

Free radicals, increased beta-secretase expression and activity, and the production of amyloid beta

Several *in vitro* studies have shown that synthetic Aβ facilitates the generation of free radicals (Hensley *et al*. 1994), causing the peroxidation of membrane lipids and the increased production of ROS in cells in culture, resulting in toxic effects from Aβ (Behl *et al*. 1994; Mattson 1995; Keller *et al*. 1997; reviewed in Reddy and Beal 2005).

In postmortem studies of the brains from AD patients and in animal models of AD, indirect markers of oxidative damage have been found to include changes in antioxidant enzymes (Pappolla *et al*. 1998; Leutner *et al*. 2000), advanced glycation end products (Wong *et al*. 2001), lipid peroxidation (Behl *et al*. 1994; Mark *et al*. 1997; Mattson *et al*. 1997; Montine *et al*. 1997; Sayre *et al*. 1997), free carbonyls (Hensley *et al*. 1994; Smith *et al*. 1996), and peroxynitration (Good *et al*. 1996; Smith *et al*. 1997). However, evidence supports alternative hypotheses regarding the deposition of Aβ and the generation of free radicals. Some researchers have suggested that oxidative stress precedes Aβ deposition (Yan *et al*. 1995; Nunomura *et al*. 2000; Pratico *et al*. 2001), and others have proposed that Aβ fibrils act as free-radical scavengers that exhibit superoxide dismutase-like activity *in vitro* (Bush *et al*. 2000). Further, the role of Aβ-associated microglia in the generation of free radicals and in the subsequent damage to mitochondria is unclear because, in AD, activated microglia are frequently observed near compact senile plaques, and the activated microglia can generate and release free radicals (Colton *et al*. 1994; Kiprianova *et al*. 1997).

The toxicity of Aβ has been clearly shown *in vitro*. When placed in a physiological solution, Aβ precipitates into fibrils and generates free radicals (Hensley *et al*. 1994). Aβ fragments have been shown to induce the generation of free radicals in cell culture (Behl *et al*. 1994; Casley *et al*. 2002) and have been found to have neurotoxic effects (Le *et al*. 1995). Recent studies have suggested that, because free radicals can promote protein cross-linking, they mediate amyloid-fibril formation, which is itself influenced by the free radical-producing amyloid peptide (Mattson 1995). Thus, a vicious cycle may result, involving the abnormal processing of APP and AB metabolism, which may be further enhanced by oxidative stress (Yan *et al*. 1995).

Recently, several studies have suggested that the activity of beta-secretase (BACE) increases in sporadic AD patients (Fukumoto *et al*. 2002; Li *et al*. 2004). It remains inconclusive whether this elevated BACE activity is related to plaque formation or Aβ production in AD brains. Recently, Li *et al*. (2004) used a sandwich ELISA assay to quantitate various Aβ species in the frontal cortex of AD brains homogenized in 70% formic acid. They found that most of the Aβ species detected in rapidly autopsied brains (< 3 h) from patients with sporadic AD were Aβ (1–16) and Aβ (1–42), as well as Aβ (36–42). To establish a link between Aβ levels and BACE, they examined BACE proteins, BASE mRNA expression, and enzymatic activity in the same region of different AD brains. They found both BACE mRNA and protein expression elevated *in vivo* in the frontal cortex. The elevation of BACE enzymatic activity in AD was found to correlate with the production of Aβ (1–16) and Aβ (1–42) in the brain of AD patients. Further, to determine whether BACE elevation was due to mutations in the BACE-coding region, they sequenced the entire open reading frame of the BACE gene in the same region from AD brains and from brains of non-demented persons, and performed allelic association analysis. They found no mutations in the open reading frame of the BACE gene. In addition, they found few changes in the BACE protein and in mRNA levels in Swedish-mutated APP-transfected cells. These findings demonstrate a correlation between Aβ loads and BACE elevation in sporadic AD patients, and they also suggest that as a consequence of the deposition of Aβ, the increase in BACE may lead to an increase in Aβ production and may enhance the deposition of Aβ plaques (Li *et al*. 2004) (see also Fig. 2).

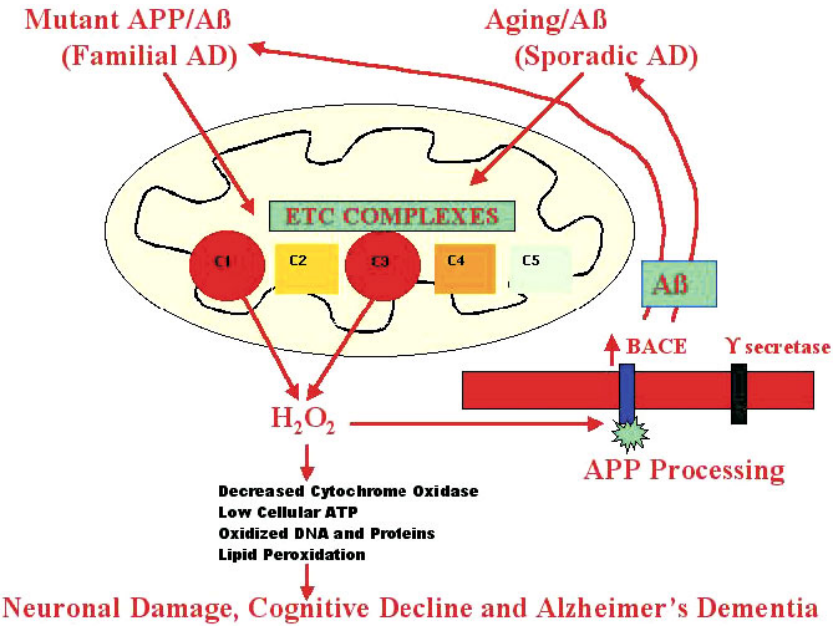

Fig. 2 A proposed model of a possible relationship between mitochondrial reactive oxygen species (ROS) and Alzheimer's disease (AD) in familial AD. In this model, mutant amyloid precursor protein (APP) and/or soluble amyloid beta (Aβ) localizes to neuronal mitochondria, which leads to the generation of free radicals, such as hydrogen peroxide (H_2O_2). The free radicals, in turn, disrupt the electron transport chain (ETC), enzyme activities, oxidized DNA, oxidized protein, and lipid peroxidation, and they inhibit cellular ATP. Based on studies of mitochondrial function in AD patients, we propose that these mitochondrial abnormalities occur selectively in learning and memory regions of the AD brain because these regions have greater oxidative stress. In this model, for sporadic AD, the free radicals that are generated due to aging activate beta-secretase (BACE) and facilitate the cleavage of the APP molecule. The cleaved APP molecule (that is, Aβ) further generates free radicals, leading to the disruption of the ETC, enzyme activities, oxidized DNA, oxidized protein, and lipid peroxidation, and to the inhibition of ATP. This feedback loop of free radicals ⇒ Aβ and Aβ ⇒ free radicals ultimately leads to neuronal damage, neurodegeneration, and cognitive decline.

Tamagno *et al*. (2002) showed that BACE is modulated by the oxidative stress product 4-hydroxynonenal (HNE). Exposure of NT2 neurons to the two classical pro-oxidant stimuli ascorbate/$FeSO_4$ and H_2O_2/$FeSO_4$ resulted in a significant generation of HNE, which was temporally followed by an increase in BACE protein levels. HNE-mediated BACE induction was accompanied by a proportional elevation of carboxy-terminal fragments of APP. Moreover, the direct relationship between BACE induction and lipid peroxidation products was strongly confirmed by the protection exerted by a short pretreatment with alpha-tocopherol, the most important antioxidant known to prevent the formation of aldehydic end-products of lipid peroxidation, including HNE. The findings from Tamagno *et al*. (2002) support their hypothesis that oxidative stress and Aβ production are strictly interrelated events, and their findings suggest that the inhibition of BACE may have a therapeutic effect that is synergic with antioxidant compounds. These studies suggest that an age-dependent increase of ROS may be directly responsible for BACE activation and Aβ production in sporadic AD patients.

In familial AD, recent molecular and cellular studies suggest that Aβ induces free radicals and promotes mitochondrial dysfunction. Casley *et al*. (2002) investigated the connection between Aβ and mitochondrial function using a cell-culture system. They incubated rat mitochondria with Aβ, and rat mitochondria with Aβ and NO, which is known to be elevated in the brains of AD patients. They also measured the enzyme levels of tricarboxyclic acid-cycle enzyme complexes, the activity of α-ketoglutarate dehydrogenase, and the activity of pyruvate dehydrogenase. They found that Aβ significantly reduces states 3 and 4 of mitochondrial respiration and that Aβ, together with NO, further diminishes mitochondrial respiration. In addition, they found that Aβ inhibits the activities of cytochrome oxidase, α-ketoglutarate dehydrogenase, and pyruvate dehydrogenase. These results suggest that Aβ has a direct effect on mitochondrial oxidative damage due to the generation of free radicals (Casley *et al*. 2002). Kim *et al*. (2002) have shown that the addition of Aβ to isolated mitochondria from brain tissues taken from rat directly induce the release of cytochrome *c* and the swelling of mitochondria. The findings from the study by Kim *et al*. (2002) suggest that in AD, Aβ

may accumulate intracellularly via abnormal APP processing and that this accumulation may exert neurotoxicity by interacting with mitochondria, oxidative damage, and apoptosis.

Overall, in AD neurons, free radicals are likely generated in the mitochondrial matrix, in both sides of the inner membrane of mitochondria and in the outer mitochondrial membrane (Fig. 2). However, it is not clearly understood how Aβ induces free radicals, and it also remains unclear what the precise sites (complex 1 or 3, or the TCA cycle) of free radical production are in the mitochondria. These critical issues are under intense investigation in many laboratories.

Amyloid beta enters mitochondria, induces the generation of free radicals, and disrupts mitochondrial function

How does Aβ induce free radicals? An obvious answer is that mutant APP and soluble Aβ enter mitochondria and disrupt the ETC. Indeed, recently several studies have reported that mutant APP and soluble Aβ do enter mitochondria and induce free radicals.

To determine whether mitochondria are critical for cellular toxicity induced by Aβ in cells, Cardoso *et al*. (2001) investigated the effect of the Aβ peptides (25–35 and 1–42) in NT2 cells with mitochondria (NT2 -*P*+) and without mitochondria (NT2 -*P*0). These investigators incubated Aβ (25–35) (1 μM) and Aβ (1–42) (10 μM) with NT2 -*P*+ and NT2 -*P*0 cells for 24 h and observed a decrease in cell viability in NT2 -*P*+ cells, but that the viability of NT2 -*P*0 cells was maintained. Further, mitochondrial membrane potential, enzyme activities, and ATP levels were decreased in NT2 -*P*+ cells treated with Aβ (25–35) but not in NT2 -*P*0 cells treated with Aβ (25–35), suggesting that Aβ peptides require functional mitochondria to induce cell toxicity (Cardoso *et al*. 2001). It is now well established that mitochondria are the major source of free radicals. There is a strong possibility that Aβ enters mitochondria, induces free radicals, disrupts the ETC, and ultimately causes mitochondrial dysfunction.

Recently, Anandatheerthavarada *et al*. (2003) studied the relationship between mutant APP and mitochondrial dysfunction in neuronal cells of Tg2576 mice. They demonstrated that by virtue of its chimeric NH_2-terminal signal, APP is targeted to mitochondria of cortical neuronal cells in select regions of the Tg2576 brain. The positively charged residues at 40, 44, and 51 of the APP molecule were found to be critical components of the mitochondrial-targeting signal. Using chemical cross-linking, together with immuno-electron microscopy, Anandatheerthavarada *et al*. (2003) showed that mitochondrial APP exists in the N-terminal transmembrane and is in contact with mitochondrial translocase proteins. In mutational studies, they also showed that the acidic domain, which spans sequence 220–290 of the APP molecule, causes a transmembrane arrest with the carboxy terminal 73-kDa portion of the protein facing the cytoplasmic side. Further, the accumulation of full-length APP in the mitochondrial compartment in a transmembrane-arrested form caused mitochondrial dysfunction and impaired energy metabolism (Anandatheerthavarada *et al*. 2003).

Recently, Lustbader *et al*. (2004) demonstrated that Aβ-binding alcohol dehydrogenase (ABAD) is a direct molecular link between Aβ and mitochondrial toxicity. They found that Aβ interacts with ABAD in the mitochondria of AD patients and transgenic mice. The crystal structure of ABAD showed substantial deformation of the active site, preventing the binding of nicotinamide adenine dinucleotide. An ABAD peptide specifically inhibited the interaction between ABAD and Aβ, and suppressed the Aβ-induced the generation of free radicals and apoptosis in neurons. Furthermore, transgenic mice that over-expressed ABAD when crossed with mice that also over-expressed Aβ showed exaggerated neuronal oxidative stress and impaired memory. These findings suggest that ABAD and Aβ directly interact with mitochondria in AD and that this interaction may promote the leakage of ROS, mitochondrial dysfunction, and cell death, all of which may potentially underlie the mechanism of Aβ-induced mitochondrial toxicity in AD progression (Lustbader *et al*. 2004).

Crouch *et al*. (2005) studied the inhibitory potential of the Aβ (1–42) on the activity of ETC enzyme complexes in human mitochondria. They found that synthetic Aβ (1–42) specifically inhibits the terminal complex cytochrome *c* oxidase in a dose-dependent manner that is dependent on the presence of Cu^{2+} and the specific 'aging' of the Aβ (1–40) solution. Maximal cytochrome *c* oxidase inhibition occurred when Aβ (1–42) solutions were used after aging for 3–6 h at 30°C. The level of Aβ (1–42)-mediated cytochrome *c* oxidase inhibition increased as the Aβ (1–42) solutions aged, up to approximately 6 h, and then the level declined progressively as the Aβ (1–42) solutions aged to 48 h. Photo-induced cross-linking of unmodified proteins followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis revealed dimeric Aβ as the only Aβ species to provide significant temporal correlation with the observed cytochrome *c* oxidase inhibition. Analysis of brain and liver tissues from an AD transgenic mouse (Tg2576 mouse line) revealed extensive Aβ immunoreactivity within the mitochondrial fraction of the brain. These data indicate that endogenous Aβ is associated with brain mitochondria and that Aβ (1–42), possibly in its dimeric conformation, is a potent inhibitor of cytochrome *c* oxidase, but only when in the presence of Cu^{2+}. Crouch *et al*. (2005) conclude that the Cu^{2+}-dependent, Aβ-mediated inhibition of cytochrome *c* oxidase may be an important contributor to the neurodegeneration process in AD.

Does mitochondrial dysfunction trigger mitochondrial gene expression?

There are several lines of evidence to support the hypothesis that abnormal mitochondrial gene expression is initiated by mitochondrial dysfunction in AD patients. To understand the role of abnormalities in the mitochondrial ETC in AD pathogenesis, several studies have investigated mtRNA expression of mitochondrial-encoded genes in complexes 1 and 4 (Chandrasekaran *et al*. 1994, 1996, 1997; Simonian and Hyman 1994) and mtRNA expression of nuclear-encoded mitochondrial genes in complexes 4 and 5 (Chandrasekaran *et al*. 1994, 1997; Simonian and Hyman 1994). These studies suggest that decreased mtRNA expression may be due to mitochondrial DNA (mtDNA) mutations in AD patients.

To determine the role of mitochondrial abnormalities in AD, using *in situ* hybridization, electron microscopy, and immunohistochemistry techniques, Hirai *et al*. (2001) studied postmortem brain specimens from AD patients and healthy control subjects. They found increased oxidative damage in AD patients, a striking and significant increase in mtDNA in pyramidal neurons, and cytochrome oxidase in the neuronal cytoplasm, suggesting that the over-expression of mtRNA may be the result of excess mtDNA replicating selectively in neurons that undergo oxidative damage. Further, Hirai *et al*. (2001) found that the increase of mtDNA and the increase of mitochondrial proteins in the brains of AD patients were not due to an increase in morphologically normal mitochondria, but rather due to an accumulation of products from *degraded* mitochondria.

Using quantitative real time PCR, Manczak *et al*. (2004) investigated mtRNA expressions in mitochondrial-encoded genes responsible for the ETC in brain specimens from early AD and definite AD patients. Using immunofluorescence techniques, they found differentially expressed mitochondrial genes – NADH 15 kDa subunit (complex 1), cytochrome oxidase subunit 1 (complex 4), and ATPase delta-subunit (complex 5) – in the same brain sections from AD patients and control subjects. They also found a down-regulation of mitochondrial genes in complex 1 of the ETC in both early and definite AD brain specimens. In addition, they determined that the decrease of mtRNA fold changes was higher for subunit 1 compared to all other subunits that they studied, suggesting that subunit 1 is critical for the ETC. Contrary to the down-regulation of genes in complex 1, complexes 3 and 4 showed increased mtRNA expression in the brain specimens from both early and definite AD patients, suggesting that decreased cytochrome *c* oxidase places an increased demand on energy production by increasing mtRNA expression of the complex 4 subunit. Based on these results, Manczak *et al*. (2004) propose that an increase in cytochrome *c* oxidase gene expression might be the result of functional compensation by the surviving neurons.

Several researchers reported that cytochrome *c* oxidase activity was decreased in platelets and brains specimens from AD patients (Parker *et al*. 1990; 1994; Kish *et al*. 1992; Bosetti *et al*. 2002). The connection between decreased cytochrome *c* oxidase and increased mtRNA has been cautiously interpreted as a compensatory mechanism in AD (Reddy and Beal 2005). It is possible that, to compensate for the loss of cytochrome *c* oxidase activity (caused by the disruption of the ETC), mtRNA from mitochondrial-encoded genes is activated in the surviving brain neurons of AD patients. The loss of mitochondrial function and the immediate compensation of mtRNA may likely be simultaneous events in AD patients. Decreased cytochrome *c* oxidase activity is well-established in the AD literature (Parker *et al*. 1990; 1994; Kish *et al*. 1992; Bosetti *et al*. 2002), but only a few studies have reported compensatory mechanisms in AD (Hirai *et al*. 2001; Strazielle *et al*. 2003; Manczak *et al*. 2004; Reddy *et al*. 2004).

The role of aging in the progression of Alzheimer's disease

The free-radical theory of aging, a prominent aging hypothesis, holds that during aging, an increase in ROS in mitochondria causes mutations in the mtDNA and damages mitochondrial components, resulting in senescence (Manczak *et al*. 2005). Findings from gene expression studies (Lu *et al*. 2004; Reddy *et al*. 2004), mtDNA studies (Lin *et al*. 2002), and aging animal-model studies (Trifunovic *et al*. 2004; Manczak *et al*. 2005; review by Melov 2004) also support this hypothesis, which, taken together, suggest that an age-dependent increase in ROS may be a factor leading to age-related diseases, such as sporadic AD, which is a late-onset disease. However, current knowledge of early cellular changes in AD progression is limited, particularly for sporadic AD, which is the form that involves the vast majority of AD patients. Studies of familial AD (early onset) and of recent *in vivo* and *in vitro* models of AD suggest that mutations in APP, presenilin 1, and presenilin 2 may activate beta and gamma secretases and may cleave 40–42 residues (or 4 kDa) of the carboxy terminus of APP. The cleaved Aβ may lead to a sequence of events, including the generation of free radicals, oxidative damage, mitochondrial dysfunction, and inflammation in the progression of disease in AD patients. However, if this process is found to be the case in patients with sporadic AD, its initial trigger(s) is still not known. Although studies of AD progression have been based on models of familial AD, they may also provide information applicable to sporadic AD, where an age-dependent increase of ROS has also been identified as a possible key factor leading to disease progression (Reddy and Beal 2005).

A challenging question is how mitochondrial ROS could lead to AD progression. Based on findings from our laboratory (Manczak *et al*. 2004, 2005; Reddy *et al*. 2004)

and others (Cardoso *et al*. 2001; Lustbader *et al*. 2004; Crouch *et al*. 2005), we propose a model (Fig. 2) of a possible relationship between mitochondrial ROS and AD. In this model, mutant APP or soluble Aβ localizes to neuronal mitochondria (Anandatheerthavarada *et al*. 2003; Lustbader *et al*. 2004; Crouch *et al*. 2005; Reddy *et al*. unpublished observations), which leads to the generation of free radicals (such as H_2O_2) (Takuma *et al*. 2005). The free radicals, in turn, disrupt the ETC, enzyme activities, oxidized DNA, oxidized protein, lipid peroxidation, and inhibited cellular ATP (Takuma *et al*. 2005). Based on studies of mitochondrial function in AD patients (see review by Reddy and Beal 2005), we suggest that this chain of events occurs selectively in learning and memory regions of the AD brain because these regions have greater oxidative stress (less oxidative defenses and more free radicals). In this model, the free radicals that are generated activate secretases and facilitate the cleavage of the APP molecule. The cleaved APP molecule (that is, Aβ) further generates free radicals, leading to the disruption of the ETC, enzyme activities, oxidized DNA, oxidized protein, and lipid peroxidation, and to the inhibition of ATP. This feedback loop of free radicals ⇒ Aβ, and Aβ ⇒ free radicals ultimately leads to neuronal damage, neurodegeneration, cognitive decline, and devastating symptoms for AD patients.

Can mitochondrially targeted antioxidants protect neurons?

The recent work by Schriner *et al*. (2005) clearly demonstrates that mitochondrially targeted catalase decreases free radicals (mainly H_2O_2), leads to reduced mitochondrial oxidative damage, and increases the lifespan of catalase transgenic mice. To determine the role of catalase in mitochondrial function, they created an AD transgenic mouse line that over-expresses human catalase localized to peroxisomes, nuclei, and mitochondria, in order to study the effects of aging from birth to death in this mouse line. Interestingly, but not surprisingly, they found that these mice experienced an increased life-span of 5.5 months longer than the lifespan of control wild-type mice, suggesting that over-expressed catalase in mitochondria decreases ROS and boosts the functioning of mitochondria.

As previously discussed, if Aβ and aging are keys to the generation of H_2O_2 in AD neurons, then mitochondrially targeted catalase, glutathione, MitoQ (a derivative of ubiquinone targeted to mitochondria) (Kelso *et al*. 2001), or MitoVit-E (a derivative of vitamin E targeted to mitochondria) (Smith *et al*. 1999) may likely, rapidly convert toxic H_2O_2 into H_2O and O_2. This continuous conversion of H_2O_2 into H_2O and O_2 may lead to reduced oxidative damage (in DNA, proteins, and lipid peroxidation) and may maintain mitochondrial function in AD. The feedback loop of free radicals ⇒ Aβ and Aβ ⇒ free radicals appears to be preventable by antioxidant treatment that targets mitochondria in AD patients. These events may ultimately help reduce Aβ production and mitochondrial toxicity in the neurons of AD patients, may increase O_2 consumption, and may help increase the life-span of AD patients.

Recently, several molecular and cellular studies of *in vitro* and *in vivo* animal models of AD reported that mitochondrial antioxidant treatments have beneficial effects on AD patients. A large body of data suggests that free radical oxidative damage – particularly of neuronal lipids (Lovell *et al*. 1995; Markesbery 1998; Markesbery and Carney 1999), proteins (Lyras *et al*. 1998; Butterfield *et al*. 2001), and nucleic acids (Lyras *et al*. 1998) – is extensive in the brains of AD patients. Increased oxidative stress is thought to result in the generation of ROS, which is reported to be released by microglia that are activated by Aβ (Qin *et al*. 2002).

In vivo models of antioxidant treatments of Alzheimer's disease

Using a Tg2576 mouse model of AD and treating the Tg2576 mice with a vitamin E-supplemented diet, *in vivo* studies reported decreased Aβ 1–40 and Aβ 1–42 levels, and in another study, the administration of vitamin E in a transgenic mouse model of tau pathology ameliorated tau aggregates (Nakashima *et al*. 2004), suggesting that vitamin E may have a direct effect on AD pathology (Conte *et al*. 2004; Sung *et al*. 2004). The administration of Curcumin to Tg2576 mice also showed encouraging results in reducing both oxidative damage and Aβ deposition (Lim *et al*. 2001; Yang *et al*. 2005). Further, melatonin reduced brain levels of Aβ, abnormal protein nitration, and increased the life span of Tg2576 mice (Matsubara *et al*. 2003). A synthetic superoxide, dismutase catalase mimetic prevented cataracts in Tg2576 mice (Melov *et al*. 2005). In addition, other studies showed the beneficial effects from several other antioxidants, gingko, and alpha lipoic acid that were added as supplements to the water or the diet of AD transgenic mice (Hagen *et al.* 2002; Stackman *et al*. 2003; Feng *et al*. 2004). These therapies were found to be safe and to result in no adverse effects.

In vitro models of antioxidant treatments in Alzheimer's disease

Using *in vitro* cell culture and AD transgenic mice of AD, several laboratories around the world are currently developing antioxidant therapies for AD patients. *In vitro* models of AD have conferred protection against Aβ-induced toxicity in various cell culture systems. Curcumin extracts have prevented Aβ fibril formation (Ono *et al*. 2004) and have protected PC12 cells (Park and Kim 2002) from insults caused by Aβ oligomers and fibrils. For example, in brain sections from Tg2576 mice, curcumin effectively blocked

Aβ 1–40 aggregation, Aβ 1–40 fibrils, and the formation of oligomers.

Antioxidant clinical trials using Alzheimer's disease patients

Of the several clinical trials conducted thus far to use antioxidants in the treatment of AD patients, only eight studies were judged adequate in terms of appropriateness of experimental design (Ahlemeyer and Krieglstein 2003). Among these eight, seven studies showed positive effects of the antioxidant EGb 761. However, even in the relatively widely investigated EGb 761, only a few clinical trials specifically focused on AD patients (Howes and Houghton 2003; Schulz 2003). In a randomized, double blind, placebo-controlled study, Le Bars (2003) reported that AD patients who were administered EGb 761 (240 mg/day) for 52 weeks showed improvements in visual constructional impairment, a lesser degree of worsening in verbal deficits, and an improvement (although minimal) in both visual and verbal deficits. Similarly, AD patients with presenile and senile primary degenerative dementia and multiinfarct dementia from mild to moderate severity showed cognitive improvements when treated with EGb 761 (Kanowski and Hoerr 2003). In contrast, AD patients (66–76 years of age) who were treated with EGb 761 (240 or 160 mg/day) for 24 weeks showed no improvement in vascular dementia or in age-associated memory impairment compared to AD patients treated with placebos (van Dongen *et al.* 2003).

In addition, several recent antioxidant studies using AD patients revealed beneficial effects of diets supplemented with vitamin E (Morris *et al*. 1998, 2002; Grundman *et al*. 2004). The administration of vitamin E and vitamin C supplements, in combination, was found to be associated with a reduced prevalence and incidence of AD in an elderly population (Zandi *et al*. 2004). Recently, Morris *et al*. (2005) examined whether food intake of vitamin E, alpha-tocopherol equivalents (a measure of the relative biologic activity of tocopherols and tocotrienols), or individual tocopherols protect against incident AD and cognitive decline. They observed that higher intakes of vitamin E and alpha-tocopherol equivalents were associated with a reduced incidence of AD in an elderly population, suggesting that antioxidant treatments at the early onset of disease are promising and may be effective in delaying AD progression (Morris *et al*. 2005). However, the pathological effects of oxidative stress are yet to be assessed in patients or elderly individuals treated with antioxidants.

In another clinical study, to determine the neuroprotective effects of cholinesterase inhibition and oxidative stress in AD patients, huperzine A was administered to AD patients in daily doses of 300 mg/day for the first 2–3 weeks of drug administration and then 400 mg/day for the next 4–12 weeks. These AD patients exhibited significant improvement in their cognitive, non-cognitive, and ADL functions (Zhang *et al*. 2003). In addition, in placebo-controlled, double-blind, randomized clinical trials, *Melissa officinalis* and *Salvia officinalis* were administered to patients with mild and moderate AD, with their cognitive functions also significantly improved (Akhondzadeh *et al*. 2003a, 2003b). Thus far, these huperzine A clinical trials have been conducted mostly in China. However, recently in the United States, to determine the effectiveness of huperzine A on AD patients, the NIH National Institute on Aging and Alzheimer's Disease Cooperative Study have collaboratively initiated a Phase II clinical trial. More recently, the John Douglas French Foundation Institute for the Study of Aging has initiated a Phase II clinical trial using curcumin on AD patients. These initial clinical trials may eventually become precursors for antioxidant clinical trials for AD patients.

Concluding remarks

Recent advances in molecular, cellular, and animal model studies of AD have revealed that mutant APP, Aβ, and other APP derivatives are key factors in the generation of free radicals and oxidative damage in AD development and progression. In the last three decades, tremendous progress has been made in mitochondrial research, and a wide body of evidence has emerged that provides clues to link mitochondrial oxidative damage and AD development and progression. Several molecular and cellular studies revealed that Aβ enters mitochondria and induces the generation of free radicals, which leads to oxidative damage in AD neurons. Researchers in the AD field are beginning to recognize the involvement of mutant APP and its derivatives in AD progression and also the role of Aβ in mitochondrial oxidative damage. In treating AD patients, currently available FDA-approved drugs [Tacrine (Cognex®), Donepezil (Aricept®), Rivastigmine (Reminyl®) and Namenda®)] provide temporary relief from dementia, but they are frequently associated with adverse drug effects and do not modify AD pathology. There is an urgent need for developing alternative approaches to AD therapeutics. Recently, several antioxidants have been systematically tested in animal and cell models of AD and, to lesser extent, in clinical trials. Antioxidants were found to be safe, had few adverse effects, readily crossed the blood brain barrier, and, to a certain extent, improved cognitive functions of AD patients. Thus, antioxidants appear to be a promising mitochondrial medicine to treat AD patients.

Acknowledgements

The author thanks Sandra Oster, PhD, Neurological Sciences Institute, Oregon Health & Science University, for editing the review. This research was supported, in part, by the American Federation for Aging Research and NIH Grant #AG22643.

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