

Review Article

Mitochondrial Oxidative Damage in Aging and Alzheimer's Disease: Implications for Mitochondrially Targeted Antioxidant Therapeutics

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The overall aim of this article is to review current therapeutic strategies for treating AD, with a focus on mitochondrially targeted antioxidant treatments. Recent advances in molecular, cellular, and animal model studies of AD have revealed that amyloid precursor protein derivatives, including amyloid beta ($A\beta$) monomers and oligomers, are likely key factors in tau hyperphosphorylation, mitochondrial oxidative damage, inflammatory changes, and synaptic failure in the brain tissue of AD patients. Several therapeutic strategies have been developed to treat AD, including anti-inflammatory, antioxidant, and anti-amyloid approaches. Among these, mitochondrial antioxidant therapy has been found to be the most efficacious in reducing pathological changes and in not producing adverse effects; thus, mitochondrial antioxidant therapy is promising as a treatment for AD patients. However, a major limitation in applying mitochondrial antioxidants to AD treatment has been the inability of researchers to enhance antioxidant levels in mitochondria. Recently, however, there has been a breakthrough. Researchers have recently been able to promote the entry of certain antioxidants—including MitoQ, MitoVitE, MitoPBN, MitoPeroxidase, and amino acid and peptide-based SS tetrapeptides—into mitochondria, several hundred-fold more than do natural antioxidants. Once in the mitochondria, they rapidly neutralize free radicals and decrease mitochondrial toxicity. Thus, mitochondrially targeted antioxidants are promising candidates for treating AD patients.

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INTRODUCTION

Alzheimer's disease (AD) is a complex, multifactorial, heterogeneous mental illness, which is characterized by an age-dependent loss of memory and an impairment of multiple cognitive functions. AD is associated with the presence of intracellular neurofibrillary tangles (NFTs) and extracellular amyloid beta ($A\beta$) plaques, the loss of neuronal subpopulations, mitochondrial oxidative damage, synaptic loss, and the proliferation of reactive astrocytes and microglia [1]. With the life span of humans increasing and with decreasing cognitive function in elderly individuals with AD-related dementia, AD has become a major health problem in society. Therapeutic interventions are urgently needed to minimize the ill effects of this devastating disease. Genetic mutations are responsible for causing early onset "familial" AD (constituting only 2% of AD cases), but the causal factor(s) for the vast majority of late-onset "sporadic" AD cases is still unknown. In addition, cellular changes in AD neurons that occur in late-onset AD are also unknown. This article

describes cellular changes in AD progression and AD therapeutic strategies, and then focuses on mitochondrially targeted antioxidants as potential therapies for AD.

HISTOPATHOLOGICAL AND CELLULAR CHANGES IN AD PROGRESSION

Histological, pathological, molecular, cellular, and gene expression studies of AD have revealed that multiple cellular pathways are involved in AD progression [2, 3]. Pathologically, there are no differences between early- and late-onset AD [4]. In patients with late-onset AD, pathological changes—including $A\beta$ production and deposits, NFTs, synaptic damage, and neuronal loss—occur later than in patients with early-onset AD [1, 4–6]. In contrast to early-onset AD in which genetic mutations accelerate the disease process, in late-onset AD, in the absence of genetic mutation, age-related cellular changes control AD progression [6]. Therefore, late-onset AD takes more time to develop [4]. Much

research has been done on early-onset AD in terms of pathophysiology and cellular changes that regulate AD progression, but we still need more research to understand causal factors, pathophysiology, and cellular changes that are responsible for disease development and progression in late-onset AD.

Several factors are known to be involved in the development of late-onset AD, with two of the major ones being aging [1] and mitochondrial abnormalities [5–9]. Other contributing factors are the ApoE genotype [10, 11], insulin-dependent diabetes [12], and environmental conditions, including diet [13].

In early-onset AD, recent molecular, cellular, and animal-model studies have provided evidence that a 4 kd peptide, a cleavage product of amyloid precursor proteins (APP) due to cleavage of β and γ secretases, is a key factor in AD development and progression [4]. The formation of the 4 kd $A\beta$ peptide in the brains of AD patients is a progressive and sequential process. Initially, soluble monomeric and oligomeric forms of 40–42 amino acid residues accumulate and later become insoluble fibrils and $A\beta$ deposits. Recent cellular and molecular studies of triple AD transgenic mice suggested that $A\beta$ production in early-onset AD may facilitate tau pathology [14–16]. The $A\beta$ plaques in the transgenic mice were also found to be associated with activated microglia and astrocytes and to trigger inflammatory responses [17]. In addition, recent molecular, cellular, and animal model studies have revealed that mutant APP and $A\beta$ enter mitochondria and interact with an $A\beta$ -induced alcohol dehydrogenase (ABAD) protein, disrupt the electron transport chain (ETC), generate reactive oxygen species (ROS), free radicals derived from molecular oxygen in the mitochondria, and inhibit the generation of cellular adenosine triphosphate [5, 6, 18, 19]. These results suggest that mutant APP and mutant $A\beta$ interacting with mitochondrial proteins cause mitochondrial dysfunction in early-onset AD [5, 6, 18].

THERAPEUTIC STRATEGIES

Recent cellular and animal model studies revealed that AD progression involves such cellular changes as inflammatory responses, mitochondrial oxidative damage, synaptic failure, and hyperphosphorylation of tau, all of which are directly related to aging and $A\beta$ production [13–16, 20–24]. Based on cellular, histopathological, and behavioral changes observed in postmortem brains of late-onset AD patients and in AD transgenic mouse models, several therapeutic strategies have been developed to treat AD patients, including immunotherapy [15, 25–28], anti-inflammatory therapy [29–32], antioxidant therapy [33–43], cholinergic therapy [44–51], cell cycle therapy [8, 52–54], and hormonal therapy [55–57]. This article briefly discusses four of these major therapeutic approaches, with special emphasis on mitochondrially targeted antioxidants.

IMMUNOTHERAPY

Current immunotherapeutic strategies are aimed at decreasing $A\beta$ levels in late-onset AD patients by inhibiting $A\beta$

generation [58], reducing soluble $A\beta$ levels [59, 60], and enhancing $A\beta$ clearance from the brain [15, 26–28].

Immunization of $A\beta$ in AD transgenic mouse models has shown that $A\beta$ levels can be reduced in the brains of AD mice [14, 25–27]. With encouraging results from in vivo studies that have aimed at abolishing $A\beta$ deposits in cellular and animal models of AD, Elan Pharmaceuticals moved quickly to investigate, in phase II clinical trials, immunotherapy to reduce $A\beta$ [25]. However, this research was stopped because AD subjects developed symptoms of aseptic meningoencephalitis [25]. The following critical issues need to be addressed before resuming immunotherapy clinical trials using AD subjects: (1) the long-term consequences of $A\beta$ immunization for the AD brain need to be identified, (2) even though immunotherapy has been found to clear $A\beta$ deposits in the AD brain, the downstream effects of AD progression still need to be determined, and (3) the relationship between the clearing of $A\beta$ and the improvement of cognitive function in AD patients needs to be clarified. Currently, several laboratories are actively involved in immunotherapy research to clear both soluble and insoluble $A\beta$ from the brain and to improve behavioral changes in AD transgenic mice.

ANTI-INFLAMMATORY THERAPY

Inflammation of brain tissue is an important component in the pathogenesis of AD, involving the activation of both microglia and astrocytes. Recent histological studies have revealed the presence of activated microglia and reactive astrocytes in and around extraneuronal $A\beta$ plaques in brains from AD patients. These activated microglia and reactive astrocytes are believed to facilitate the clearing of $A\beta$ deposits from the brain parenchyma [13]. However, now there is increasing evidence to suggest that the chronic activation of microglia, presumably via the secretion of cytokines and reactive molecules [61, 62], may exacerbate $A\beta$ plaque pathology as well as enhance the hyperphosphorylation of tau and the formation of NFTs [14–16]. Thus, the suppression of microglial activity in the AD brain has been considered a possible therapeutic strategy to treat AD patients [13, 30]. Suppressing anti-inflammatory drugs, particularly nonsteroidal anti-inflammatory drugs, have been found to lessen the effects of $A\beta$ in transgenic mice [29–32].

CHOLINERGIC THERAPY

AD affects cholinergic neurotransmission in the neurons of the basal forebrain [63]. There is increasing evidence that the enzymes involved in the synthesis of choline acetyltransferase (ChAT) and the degradation of acetylcholine (ACh) may be responsible for deficits in cholinergic neurotransmission. Strategies to boost the levels of ACh in the AD brain are being developed to treat AD patients, to reduce NFTs and $A\beta$ levels, and to improve cognition. Similar to ChAT, butyrylcholinesterase (BChE) inactivates the neurotransmitter ACh and, thus, is a viable therapeutic target for AD. Greig et al [47] tested potent, reversible, and brain-targeted BChE inhibitors (cymserine analogs). In rats, cymserine

analogs caused long-term inhibition of brain BChE and elevated extracellular ACh levels, without inhibiting ChAT [47]. In slices from rat brains, selective BChE inhibition augmented long-term potentiation. These compounds (cymserine analogs) improved the cognitive performance (maze navigation) of aged rats. In cultured human SK-N-SH neuroblastoma (N2a) cells, BChE inhibitors reduced intra- and extracellular A β precursor proteins and secreted A β peptides without affecting cell viability. Cholinergic treatment of transgenic mice overexpressing human mutant APP also resulted in lower levels of A β peptides in their brains than the levels of A β peptides in the brains from healthy (control) rats. Selective, reversible inhibition of brain BChE may be a viable treatment for AD to improve cognition and to modulate neuropathological markers of the disease. Discoveries in cholinergic therapeutics have already led to the development of several cholinesterase inhibitors [44–51]. Four cholinesterase-inhibiting drugs are currently being prescribed for patients with mild to moderate AD: Donepezil, Rivastigmine, Galantamine, and Tacrine. However, these drugs provide only temporary relief of AD symptoms, and there is no evidence as yet to suggest that they reduce AD pathology. As a result, cholinergic therapy is considered a short-term intervention for certain symptoms of AD, since it is still unknown whether cholinesterase inhibitors modify A β pathology in AD mouse models and in AD patients. This issue has been under intense investigation in many laboratories across the world.

OXIDATIVE STRESS AND ANTIOXIDANT THERAPY

Oxidative stress is a major factor associated with the development and progression of AD and other forms of dementia. A large body of data suggests that free radical oxidative damage—particularly of neuronal lipids [64, 65], nucleic acids [23, 24, 66–68], and proteins [66, 67, 69, 70]—is extensive in the brains of AD patients. Increased oxidative stress is thought to result in the generation of free radicals and ROS, which is reported to be released by microglia activated by A β [71, 72]. Compared to other organs, the brain has been found to be more vulnerable to oxidative stress due to its high lipid content, its relatively high oxygen metabolism, and its low level of antioxidant defenses [70, 73, 74]. Markers of oxidative stress, such as 8-hydroxyguanosine and hemeoxygenase, have been localized to pathologic lesions in the brains of AD patients [75–78].

Using PC 12 cells and A β (25–35) peptide, Bozner et al studied the connection between A β and mitochondrial DNA damage. They exposed PC 12 cells to an A β (25–35) in frame and scrambled at 50 mM concentration for 24 hours to 50 hours. Oxidative damage of mitochondrial DNA was assessed using a Southern blot technique and a mitochondrial DNA-specific probe recognizing a 13.5-kilobase restriction fragment. PC 12 cells exposed to A β exhibited marked oxidative damage of mitochondrial DNA as evidenced by characteristic changes on Southern blots, but not in cells exposed to the scrambled A β peptide, suggesting that A β peptide is responsible for mitochondrial DNA damage, and ultimately leading to mitochondrial dysfunction in AD [68]. Further,

evidence from a recent gene expression study [21] suggests that mutant APP or A β may generate free radicals and promote mitochondrial dysfunction, one or both of which may lead to oxidative damage. Altered levels of mitochondrial enzymes have been found to be directly responsible for a decrease in energy production in the brains of late-stage AD patients [5]. Soluble or insoluble forms of A β have been suggested to impair ATP production by generating defects in mitochondrial energy metabolism and oxidative stress [79]. Taken together, these results suggest that oxidative stress is a key event in AD pathogenesis.

FREE RADICAL PRODUCTION AND MITOCHONDRIAL OXIDATIVE DAMAGE

In the literature on AD, the terms “oxidative stress” or “oxidative damage” are commonly used to explain the balance between the production of oxidants and the endogenous antioxidant defenses in neuronal cells. In general, cells undergo apoptotic death when there is an imbalance between oxidants and antioxidants (more oxidants than antioxidant defenses). This oxidative damage mainly occurs via the mitochondrial ETC [5, 6].

Mitochondria, which are cytoplasmic organelles, are responsible for the production of cellular ATP. Mitochondria are involved in 3 important cell functions: (1) producing ATP and regulating intracellular Ca²⁺; (2) releasing proteins that activate the caspase family of proteases; and (3) altering the reduction-oxidation potential of cells [5]. Disruption of the ETC has been recognized as an early characteristic of apoptotic cell death. ETC involves the reduction of hydrogen peroxide (H₂O₂) to H₂O and O₂ by catalase or glutathione peroxidase accepting electrons donated by NADH and FADH₂, and then yielding energy for the generation of ATP from adenosine diphosphate and inorganic phosphate [6, 80–82].

The production of mitochondrial superoxide radicals (O₂^{•−}) occurs primarily at discrete points in the ETC at complexes 1 and 3 [83], and in components of tricarboxylic acid (TCA), including α -ketoglutarate dehydrogenase [84] (see Figure 1). In addition, mitochondrial O₂^{•−} are generated in the outer mitochondrial membrane. Monoamine oxidase (flavoprotein), localized on the outer mitochondrial membrane, catalyzes the oxidative deamination of primary aromatic amines. This deamination is a quantitatively large source of H₂O₂ that contributes to an increase in the steady-state concentrations of ROS within both the mitochondrial matrix and the cytosol [80]. These released H₂O₂ and O₂^{•−} are carried to the cytoplasm via voltage-dependent anion channels and, ultimately, lead to the oxidation of cytoplasmic proteins (see Figure 1). The chronic exposure of ROS to cells can result in oxidative damage to mitochondrial and cellular proteins, lipids, and nucleic acids, and acute exposure to ROS can inactivate the TCA-cycle aconitase and the iron-sulfur centers of ETC at complexes 1, 2, and 3, resulting in a shutdown of mitochondrial energy production [5, 6, 85].

The generation of free radicals can occur via several cellular insults, including ultraviolet irradiation [86], redox-cycling of quinones [86], the metabolism of xenobiotics [86],

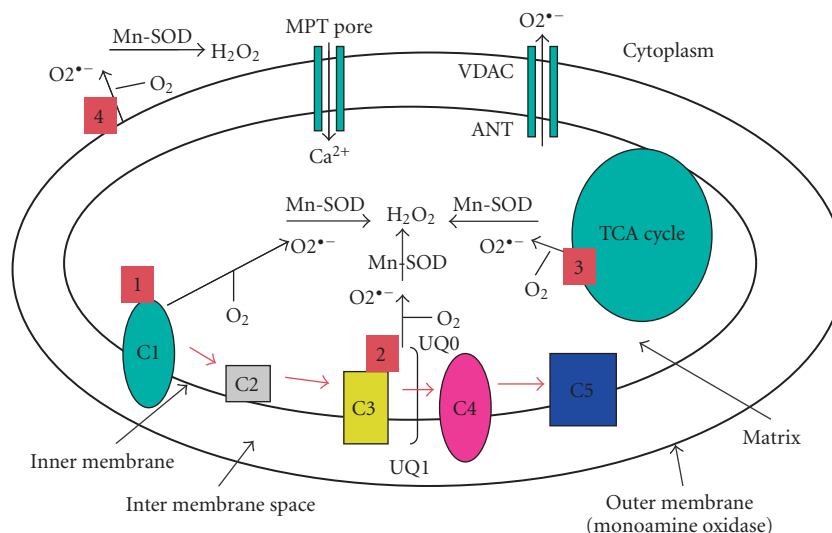


FIGURE 1: Illustration of sites of free radical generation in the mitochondria. In the respiratory chain, complexes 1 and 3 leak electrons to oxygen, producing primarily superoxide radicals (or $O_2^{\bullet-}$). The $O_2^{\bullet-}$ are dismutated by manganese superoxide dismutase to generate H_2O_2 and oxygen. Complex 1 generates $O_2^{\bullet-}$ only toward the matrix. Complex 3, on the other hand, generates $O_2^{\bullet-}$ toward both the intermembrane space and the matrix. The components of tricarboxylic acid, including α -ketoglutarate dehydrogenase, also generate $O_2^{\bullet-}$ in the matrix. Free radicals are generated in the outer mitochondrial membrane (monoamine oxidase) and catalyze the oxidative deamination of primary aromatic amines, leading to the generation of H_2O_2 .

aging [87], environmental mitochondrial toxins [88–90], and mutant toxic proteins (eg, $A\beta$ in AD, mutant huntingtin in Huntington's disease, alpha-synuclein in Parkinson's disease, mutant SOD1 in amyotrophic lateral sclerosis) [5, 8, 87].

FREE RADICALS, ABNORMAL PROCESSING OF APP, AND $A\beta$ METABOLISM

There is mounting evidence to suggest that in late-onset AD, age-related free radicals, which are generated in the mitochondria, are carried to the cytoplasm where they activate beta secretase and facilitate the cleavage of the APP molecule [6]. The cleaved APP molecule (ie, $A\beta$) further generates free radicals, leading to the disruption of the ETC and enzyme activities, the inhibition of ATP, and the subsequent oxidation of both nuclear and mitochondrial DNA proteins. The damage caused by mitochondria ultimately leads to neuronal damage, neurodegeneration, and cognitive decline in AD patients [6].

AGE-DEPENDENT MITOCHONDRIAL Ca^{2+} AND OXIDATIVE DAMAGE

The dysregulation of age-related Ca^{2+} and an increased production of ROS may contribute to late-onset neurodegenerative disorders such as AD. These alterations are often attributed to impaired mitochondrial function, yet few studies have directly examined isolated mitochondria from various regions of the aged brain. Recently, Brown et al [91] examined Ca^{2+} influx and ROS production in isolated mitochondria from Fischer 344 rats ranging in age from 4 to

25 months. Isolated mitochondria from the cortex of the 25-month-old rat brain exhibited greater rates of ROS production and mitochondrial swelling in response to increasing Ca^{2+} loads compared to mitochondria isolated from younger (4- and 13-month-old) rats. This increased swelling is indicative of the opening of a mitochondrial permeability transition pore, suggesting an impaired Ca^{2+} influx in aged animals (see Figure 1). These age-related differences were not observed in isolated mitochondria from the cerebellum of these rats. Together, these results suggest region-specific, age-related alterations in mitochondrial responses to Ca^{2+} [91]. In addition to aging, in age-related diseases such as AD, $A\beta$ promotes the opening of the mitochondrial permeability transition pore, through which free cytosolic Ca^{2+} enter the mitochondria (see Figure 1). The mitochondrial Ca^{2+} may disrupt the ETC, which would elevate the production of free radicals in the mitochondria [92]. To determine the extent of free radical production and oxidative damage in the spinal cord and neocortex of the rat brain, Sullivan et al [93] studied several parameters of mitochondrial physiology in the normal neocortex and spinal cord. In situ measurements revealed significantly higher levels of $O_2^{\bullet-}$ production, lipid peroxidation, and mitochondrial DNA oxidation in the spinal cord than these levels in neocortical neurons. Real-time PCR analysis of mitochondrial genes demonstrated differences in mitochondrial transcripts coupled with decreases in complex 1 enzyme activity and in respiration, in the spinal cord mitochondria. The threshold for calcium-induced mitochondrial permeability transition was substantially reduced in the spinal cord relative to the neocortex and was modulated by lipid peroxidation. These findings suggest that studying mitochondrial damage in the spinal cord may

be productive in learning about causes of age-related neurodegenerative diseases [93].

A β AND MITOCHONDRIAL FUNCTIONAL ASSOCIATION

To determine whether mitochondria are critical for cellular toxicity induced by A β , Cardoso et al [94] investigated the effects of A β peptides in NT2 cells with mitochondria (NT2 -P+) and without mitochondria (NT2 -P0) [94]. In NT2 -P+ cells, they observed a decrease in cell viability, mitochondrial membrane potential, enzyme activities, and ATP levels, but they did not find such decreases in NT2 -P0 cells, suggesting that A β peptides require functional mitochondria for the induction of cell toxicity. Further, several studies reported the association of mutant APP derivatives with mitochondria [18, 19, 95]. Anandatheerthavarada et al [95] observed that in cortical neurons, the accumulation of full-length APP in the mitochondrial compartment in a transmembrane-arrested form of APP is responsible for mitochondrial dysfunction and impaired energy metabolism [95]. Lustbader et al [18] demonstrated that A β is localized to mitochondria in the neurons of AD transgenic mice. Further, they also found that A β directly interacts with an A β -binding ABAD protein in the mitochondria, leading to oxidative damage and mitochondrial dysfunction [18]. Crouch et al [19] studied the inhibitory potential of synthetic A β (1–42) on the activity of ETC enzyme complexes in human mitochondria. They found that synthetic A β (1–42) inhibits the terminal complex cytochrome *c* oxidase in a manner that is dependent on the presence of Cu²⁺. In the Crouch study, maximal cytochrome *c* oxidase inhibition occurred when synthetic A β (1–42) solutions were used after they aged from 3 to 6 hours at 30°C. The level of A β (1–42)-mediated cytochrome *c* oxidase inhibition increased up to 6 hours after the A β (1–42) solution aged, and then the level declined progressively as the A β (1–42) solutions aged to 48 hours. These data strongly suggest that endogenous A β is associated with brain mitochondria and that synthetic A β (1–42) is a potent inhibitor of cytochrome *c* oxidase [19]. All of these studies combine to suggest that mitochondria are vulnerable to A β and/or age-related oxidative damage.

ANTIOXIDANT TREATMENT

Using a Tg2576 mouse model of AD and treating the Tg2576 mice with a vitamin E-supplemented diet, researchers in vivo studies reported decreased A β (1–40) and A β (1–42) levels [34, 35]. In another study, researchers using a transgenic mouse model of tau pathology found that the administration of vitamin E ameliorated tau aggregates [33], suggesting that vitamin E may have a direct effect on AD pathology [34, 35]. The administration of curcumin (an antioxidant) to Tg2576 mice also showed encouraging results when both oxidative damage and A β deposits were reduced [38]. Further, melatonin reduced brain levels of A β and abnormal protein nitration, and increased the life span of Tg2576 mice [36]. A synthetic superoxide (a dismutase catalase mimetic) prevented cataracts in AD transgenic mice [96].

Several recent antioxidant studies using AD patients revealed beneficial effects of diets supplemented with vitamin E [39, 40, 42]. The combined administration of vitamin E and vitamin C supplements was associated with a reduced prevalence and incidence of AD in an elderly population [43]. Morris et al [41] examined whether food intake of vitamin E, alpha-tocopherol equivalents (a measure of the relative biologic activity of tocopherols and tocotrienols), or individual tocopherols protects against AD symptoms and cognitive decline. They found that higher intake of vitamin E and alpha-tocopherol equivalents was associated with a reduced incidence of AD in an elderly population, suggesting that antioxidant treatment at the early onset of disease may be effective in delaying AD progression [41]. However, the pathological effects of oxidative stress are yet to be assessed in AD 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 (an antioxidant) was administered to AD patients in doses of 300 mg/d for the first 2–3 weeks of drug administration and then 400 mg/d for the next 4–12 weeks. At the end of 12 weeks, the AD patients exhibited significant improvement in their cognitive, noncognitive, and ADL functions [97]. These initial clinical trials may eventually become precursors for antioxidant clinical trials for AD patients.

MITOCHONDRIALLY TARGETED ANTIOXIDANT THERAPIES IN AGING AND AD

Krzepilko et al [98] studied exogenous mitochondrial antioxidants in yeast (*Saccharomyces cerevisiae*) mutants. They found that yeast mutants lacking CuZn-superoxide dismutase are hypersensitive to oxygen and have a significantly decreased replicative life span [98]. These defects can be ameliorated by the low-molecular weight exogenous antioxidant ascorbate. The effect of ascorbate on life span is complicated by its auto-oxidation in yeast cell culture media. Krzepilko and colleagues found that if negative effects of auto-oxidation are prevented by exchange of the medium, ascorbate prolongs not only the mean but also the maximal replicative life span of the yeast in an atmosphere of air and pure oxygen. The findings from this study suggest that the shortening of a healthy life span due to the lack of an antioxidant enzyme may be ameliorated by ascorbate [98].

In studies of the transgenic fruit fly *Drosophila* that overexpresses antioxidant enzymes targeted to mitochondria, Ruan et al [99] found that the antioxidant enzymes Mn-SOD (manganese superoxide dismutase) and methionine sulfoxide reductase extended the life span of *Drosophila*, suggesting that mitochondrially targeted antioxidants may reduce ROS and contribute to life span extension [99]. However, recently, Magwere et al [100] tested the effects of SOD mimetic drugs Euk-8 and -134, and the mitochondrially targeted mitoquinone (MitoQ) on life span and oxidative stress resistance of wild-type and SOD-deficient flies. They confirmed findings from other researchers that exogenous antioxidants rescue pathology associated with compromised deficiencies

to oxidative stress, but do not extend the life span of normal, wild-type flies treated with exogenous antioxidants. All three exogenous antioxidants (Mn-SOD, MitoQ, and Euk-8 and -134) led to a dose-dependent increase in toxicity in wild-type flies, an effect that was exacerbated in the presence of the redox-cycling drug, paraquat. However, important findings from this study were that in SOD-deficient flies, the antioxidant drugs increased life span. Further, the effects of these antioxidant drugs were sex-specific, and for either sex, the effects were also variable depending on (1) the stage of development at which the drugs were given, and (2) the magnitude of the dose [100].

Schriner et al [101] recently demonstrated that mitochondrially targeted catalase decreases H_2O_2 , leads to reduced mitochondrial oxidative damage, and increases the life span of catalase transgenic mice. To determine the role of catalase in mitochondrial function, they created mouse lines that overexpress human catalase localized to peroxisomes, nuclei, and mitochondria, to elucidate the effects of catalase on aging from birth to death. Catalase, found mainly in peroxisomes, rapidly converts toxic H_2O_2 into H_2O and O_2 [6]. In two independent lines of mitochondrial catalase (MCAT) mice, Schriner et al [101] found that the transgenic mice treated with MCAT expressers showed about a 20% increase in median and maximal life span (on average, 5.5 months) compared to the life span of nontransgenic, age-matched wild-type littermates. The ability of catalase to increase longevity was most apparent when the enzyme was targeted to mitochondria. Schriner et al found that the transgenic mice, which express catalase in peroxisome, had a slightly longer median life span, but showed no increase in maximal life. Nuclear catalase (NCAT) expression (in NCAT mice) had no effect on either the median life span or the maximal life span of the mice [101].

In the Schriner study [101], MCAT transgenic mice appeared to age more slowly than their age-matched littermates by several measures. While histological comparisons showed little difference between wild-type and MCAT lines in young mice (9 to 11 months old), aged transgenic mice (20 to 25 months old) had significantly less arteriosclerosis and cardiomyopathy than their wild-type littermates. Biochemical studies have shown that the slower aging found in the MCAT mice is associated with a lower level of oxidative stress and DNA damage [101]. In the Schriner study [101], H_2O_2 production by cardiac mitochondria from MCAT mice decreased 25%, and mitochondria containing catalase were protected from the toxic effects of H_2O_2 . Age-related increases in oxidative damage to total DNA and fragmentation of mitochondrial DNA were also slowed in the skeletal muscle of MCAT mice [101].

Overall, findings from these aging studies suggest that mitochondrially targeted antioxidants reduce ROS production and oxidative damage in that the flies were diseased and also contribute to healthy aging at least in laboratory mice. However, the effects of mitochondrial antioxidants in healthy humans and humans with neurodegenerative disease still need to be determined.

From these aging studies, it is clear that mitochondrially generated H_2O_2 is a critical factor in determining life span. If aging is a key to the generation of H_2O_2 in aged neurons, then mitochondrially targeted catalase, glutathione peroxidase, MitoQ [102], MitoVitE [103], MitoPBN [104], and several mitochondrially targeted peptides [105, 106] 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 reduce oxidative damage in aged neurons and may maintain mitochondrial function in the neurons of aged individuals. It has been established that an overload of Ca^{2+} induces ROS and disrupts the ETC in mitochondria. It is also possible that mitochondrially targeted antioxidants reduce ROS induced by an age-related overload of Ca^{2+} in mitochondria [91, 92]. Since age is a major factor involved in the development of late-onset AD, mitochondrially targeted antioxidants may be able to reduce oxidative damage in AD, increase O_2 consumption, help increase the life span of elderly individuals, and decrease or prevent AD progression in elderly individuals [6].

A major limitation in using antioxidant therapy to treat the age-related diseases, such as AD, has been the inability of investigators to enhance the antioxidant levels in mitochondria [107]. However, in the last 5 years, considerable progress has been made in developing mitochondrially targeted antioxidants. To increase the delivery of antioxidants to mitochondria, three types of antioxidants are being studied: triphenylphosphonium-based antioxidants, and amino acid- and peptide-based antioxidants.

HOW DO MITOCHONDRIALLY TARGETED ANTIOXIDANTS ENTER MITOCHONDRIA?

The mitochondrial ETC participates in the transfer of electrons to O_2 [6]. During this transfer, a proton gradient is generated, which drives the production of ATP by ATP-synthase [86]. ATP production in turn generates a negative potential from 150 to 180 mV across the inner mitochondrial membrane. This negative potential gradient is used to deliver lipophilic cations to mitochondria. The lipophilic cations easily permeate through the lipid bilayers and subsequently accumulate by several hundred-fold within the mitochondria, given their large mitochondrial membrane potential (see Figure 2). Using this known transfer of electrons to O_2 , Murphy and colleagues recently developed several mitochondrially targeted antioxidants: MitoQ (a derivative of mitochondrial quinoline), MitoVitE (a derivative of mitochondrially targeted vitamin E) [102, 107], and MitoPBN (a derivative of α -phenyl-N-*tert*-butyl nitron) [104]. These lipophilic cations-based antioxidants were covalently coupled to a triphenylphosphonium cation and were preferentially taken up by mitochondria [102–104]. These antioxidants initially accumulated in the cytoplasm of cells, due to a negative plasma membrane potential (see Figure 2) [102, 107, 108].

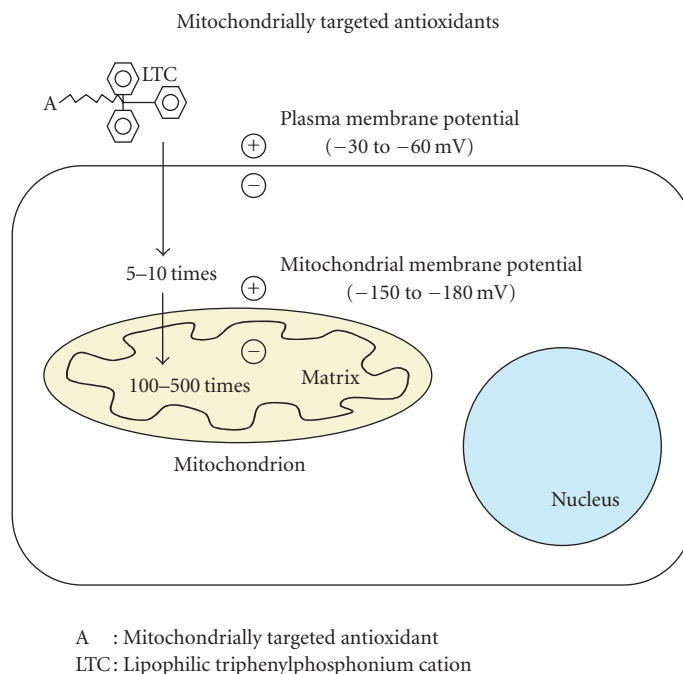


FIGURE 2: Illustration of mitochondrially targeted antioxidants. A generic mitochondria-targeted antioxidant is shown constructed by the covalent attachment of an antioxidant molecule to the lipophilic triphenylphosphonium cation. Antioxidant molecules accumulate 5- to 10-fold in the cytoplasm, which is driven by the plasma membrane potential, and then further accumulate several hundred-fold in the mitochondria.

MitoQ

MitoQ is an antioxidant consisting of two redox forms of mitochondrially targeted ubiquinone derivatives: reduced mitoquinol and oxidized mitoquinone [102]. MitoQ is attached to a phosphonium cation [10-(6-ubiquinonyl) decyltriphenylphosphonium bromide]. The molecular formula of MitoQ is $C_{37}H_{46}O_4PBr$, and its molecular weight is 665.65. MitoQ is a red, oily solid and can be stored at -20°C in the dark. It can be converted into a fully oxidized form by incubation in 95% ethanol and can be dissolved in DMSO.

MitoQ is a promising therapeutic antioxidant that has been successfully targeted to mitochondria [102]. MitoQ is a respiratory chain component buried within the lipid core of the inner membrane of mitochondria where it accepts 2 electrons from complex 1 or 2, to form the reduction product ubiquinol, which donates electrons to complex 3 [109]. The ubiquinone pool *in vivo* exists largely in a reduced ubiquinol form, acting as an antioxidant and a mobile electron transfer. Ubiquinol has been reported to function as an antioxidant by donating a hydrogen atom from one of its hydroxyl groups to a lipid peroxyl radical, thereby decreasing lipid peroxidation within the mitochondrial inner membrane [110–112]. The semiubiquinone radical formed during this process disproportionates into ubiquinone and ubiquinol [113]. The respiratory chain subsequently recycles ubiquinone back to ubiquinol, restoring its antioxidant function. MitoQ excessively accumulates in the mitochondria and converts H_2O_2 to H_2O and O_2 , and reduces toxic insults from free radicals in

the mitochondria. This reduction ultimately leads to the protection of neurons from age-related and/or disease-related mitochondrial insults in AD.

Recently, the effects of MitoQ on mitochondria in several *in vitro* cell models were tested [114–117]. In cultured fibroblasts from Friedreich Ataxia patients, MitoQ prevented cell death known to be caused by endogenous oxidative stress [115]. In a study of PC12 cells, low concentrations of MitoQ ($1\mu\text{M}$) selectively inhibited serum deprivation-induced apoptosis in PC12 cells [116]. In a study of bovine aortic endothelial cells treated with glucose/glucose oxidase and lipid peroxide, MitoQ inhibited cytochrome *c* release, caspase 3 activation, and DNA fragmentation in greater percentages than did the corresponding untargeted counterparts such as vitamin E [117]. These studies suggest that MitoQ may reduce free radicals, decrease oxidative damage, and maintain mitochondrial function. Since oxidative damage is part of the known pathophysiology of AD, there is strong interest in determining whether mitochondrially targeted antioxidants decrease oxidative damage in the neurons of AD patients [6].

Smith et al [118] studied the effects of mitochondrially targeted antioxidants, including MitoQ, on laboratory mice. Some of the mice were fed with MitoQ for several weeks until MitoQ reached steady-state concentrations within all tissues assessed, including brain, heart, liver, and kidney tissues [118]. At that point, the concentration of MitoQ in the tissues was several hundred-fold higher than that in the bloodstream. Smith et al [118] found the uptake of MitoQ to be reversible, as observed by the rapid clearance of the simple

lipophilic cation methyltriphenylphosphonium from all organs when oral administration of MitoVitE was stopped. Using mass spectrometry, they found that orally administered MitoQ entered the bloodstream and distributed in an intact active form to tissues in the brain, heart, and liver. After the mice were fed with methyltriphenylphosphonium and MitoVitE, the levels of these accumulated antioxidants were in the range of 5 to 20 nmol/g (wet weight), or about 5 to 20 μ mol/l in the tissues [118]. These high concentrations may be in a therapeutic range since MitoQ has been found to prevent oxidative damage in isolated mitochondria at lower concentrations (eg, 1 to 2.5 μ mol/l [102, 103]). Therefore, the oral administration of well-tolerated doses of MitoQ may deliver potentially therapeutic concentrations to mitochondria in vivo [118].

Further, Adlam et al [119] recently examined the effect of MitoQ in a murine model of cardiac ischemia-reperfusion injury (a mitochondrial oxidative damage model). MitoQ fed to rats significantly decreased their heart dysfunction and mitochondrial damage after ischemia reperfusion. This protection was attributed to MitoQ in the mitochondria [119].

Since natural antioxidants given at high doses without side effects have been shown to decrease oxidative damage in AD mouse models and AD patients [33–43], there is a strong rationale for testing these mitochondrially targeted antioxidants in trials using AD cell and mouse models, and AD patients.

MitoVitE

MitoVitE is an antioxidant that consists of [2-3,4-dihydro-6-hydroxy-2,5,7,8-tetra-methyl-2H-1-benzopyran-2-yl] and triphenylphosphonium bromide [86]. MitoVitE is a derivative of vitamin E that is targeted to mitochondria, and it was developed to study mitochondrial oxidative damage. MitoVitE is rapidly taken up by mitochondria, and the uptake lasts for 15 min. Accumulation ratios of 5000–6000 unit have been achieved after incubating mitochondria with 1–20 μ M MitoVitE [86]. MitoVitE is cytotoxic at 50 μ M. The effects of MitoVitE have been tested in Jurkat cells. MitoVitE was found to reduce H₂O₂-induced caspase activity [120]; to prevent oxidative stress-induced cell death in cultured fibroblasts from Friederich Ataxia patients [115]; and at (1 μ M) concentration, to inhibit cytochrome *c* release and caspase-3 activation, to inactivate complex 1, and to restore mitochondrial membrane potential and proteosomal activity in bovine aortic epithelial cells [117].

MitoPBN

MitoPBN is an antioxidant consisting of [4-[4 (1,1-dimethylethyl) oxidoimino]-methyl]phenoxy]butyl] and triphenylphosphonium bromide [104, 121]. A mitochondrially targeted analog of MitoPBN was prepared to determine the effect of ROS and mitochondrial function in mitochondria, based on a selective PBN (α -phenyl-N-*tert*-butyl nitron) reaction with carbon-centered radicals [104]. Similar to MitoQ and MitoVitE, MitoPBN was rapidly taken up by

mitochondria, with a resulting concentration ranging from 2.2 to 4.0 mM. It has been reported that MitoPBN blocks the O₂-induced activation of uncoupled proteins.

Recently, Poeggeler et al [122] developed an amphiphilic molecule, *N*-[4-(octa-*O*-acetylactobionamidomethylene)benzylidene]-*N*-[1,1-dimethyl-2-(*N*-octanoyl) amido]-ethylamine *N*-oxide, (LPBNAH, a derivative of α -phenyl-N-*tert*-butyl nitron) that exhibits profound antioxidant and neuroprotective activity and very efficiently antagonizes oxidotoxicity of primarily mitochondrial origin [122]. LPBNAH, when administered via food to rotifers, was found to decrease free radicals, to greatly enhance the survival of neurons, and to increase the life span of rotifers. The antiaging activity of LPBNAH exceeded that of other nitron compounds, such as the parent compound PBN, by at least one order of magnitude. The development of such a neuroprotective antioxidant may lead to more safe and effective treatments of age-related diseases such as AD.

AMINO ACID- AND PEPTIDE-BASED MITOCHONDRIALLY TARGETED ANTIOXIDANTS

SS tetrapeptides are aromatic cationic peptides that contain the structural motif of alternating aromatic and basic amino acid residues, along with a 2', 6'-dimethyltyrosine residue [86]. These tetrapeptides are mitochondrially targeted antioxidants. Their antioxidant properties are derived from the related compound 3, 5-dimethoxyphenol, a known phenolic antioxidant [123]. SS tetrapeptides were originally developed to develop centrally acting opioid analgesics [124, 125]. The following SS tetrapeptide compounds have been developed for mitochondrial and cellular uptake studies: (1) Dmt-D-arg-Phe-Lys NH₂ (SS-02); (2) Phe-D-Arg-Phe-Lys-NH₂ (SS-20); (3) D-Arg-Dmt-Lys-Phe-NH₂ (SS-31); and (4) Dmt-d-Arg-Phe-atnDAP-NH₂ (SS-19) [86].

SS-02 neutralizes H₂O₂ and inhibits the oxidation of linoleic acid and low-density lipoproteins. SS-31 contains same amino acid residues, as does SS-02, but in an order that exhibits antioxidant properties. SS-02 is taken up by Caco-2 cells (derived from human colorectal adenocarcinoma), and its intracellular concentration is about 10 times greater than its extracellular concentration. SS-02 and SS-19 are rapidly taken up by isolated mitochondria from mouse liver, with accumulation in the mitochondria 105-fold. The incubation of mitochondria with FCCP reduced the uptake of SS-19 by 20%, suggesting partial potential-dependent uptake [86]. Treatment of SS-02 with digitonin showed that about 85% of SS-02 is present in the mitoplast (inner membrane plus matrix).

Recently, Zhao et al [106] developed peptide antioxidants that target the inner mitochondrial membrane. These antioxidants were used to investigate the role of ROS and the mitochondrial permeable transition of 3NP in cell death caused by the peptides [t-butylhydroperoxide (tBHP) and 3-nitropropionic acid (3NP)]. The structural motif of tBHP and 3NP centers on alternating aromatic and basic amino acid residues, with dimethyltyrosine providing scavenging properties. tBHP and 3NP were found to be cell-permeable

and to concentrate at 1000-fold in the inner mitochondrial membrane. Peptide antioxidants potently reduced intracellular ROS and cell death caused by tBHP in neuronal N2a cells. These peptide antioxidants also decreased mitochondrial ROS production, inhibited mitochondrial permeability transition and swelling, and prevented cytochrome *c* release induced by Ca^{2+} in isolated mitochondria. In addition, peptide antioxidants inhibited 3NP-induced mitochondrial permeability transition in isolated mitochondria and prevented mitochondrial depolarization in cells treated with 3NP. ROS and mitochondrial permeability transition have been implicated in myocardial stunning associated with reperfusion in ischemic hearts. Peptide antioxidants were found to potently improve contractile force in an ex vivo heart model. It is noteworthy that peptide analogs without dimethyltyrosine did not inhibit mitochondrial ROS generation or swelling, and did not prevent myocardial stunning. These results clearly suggest that ROS underlies the cellular toxicity of tBHP and 3NP, and that ROS may mediate cytochrome *c* release via a mitochondrial permeability transition. Peptide antioxidants may be very beneficial in the treatment of aging and diseases associated with oxidative stress [106].

Further, Zhao et al examined the ability of a novel, cell-penetrating, mitochondrially targeted peptide antioxidant to protect against oxidant-induced mitochondrial dysfunction and apoptosis in two neuronal cell lines [105]. Treatment of neuronal cell lines with tBHP for 24 hours resulted in lipid peroxidation, significant cell death via apoptosis in both N2a and SH-SY5Y cells, phosphatidylserine translocation, nuclear condensation, and increased caspase activity. When treated with tBHP, the N2a and SH-SY5Y cells showed a significant increase in intracellular ROS, mitochondrial depolarization, and reduced mitochondrial viability. Concurrent treatment with < 1 nM SS-31 significantly decreased intracellular ROS, increased mitochondrial potential, and prevented tBHP-induced apoptosis.

The remarkable potency of SS-31 can be explained by its extensive cellular uptake and selective partitioning into mitochondria. Intracellular concentrations of [3H]SS-31 were 6-fold higher than extracellular concentrations. Studies using isolated mitochondria revealed that [3H]SS-31 was concentrated approximately 5000-fold in the mitochondrial pellet. By concentrating in the inner mitochondrial membrane, SS-31 became localized to the site of ROS production, and protected against mitochondrial oxidative damage and against further ROS production. SS-31 represents a novel platform for mitochondria-targeted antioxidants with broad therapeutic potential [105].

CONCLUSIONS

Recent advances in molecular, cellular, and animal model studies have revealed that mitochondria are the major source of free radical generation and of oxidative damage in aging and age-related neurodegenerative diseases. It is possible that age-related mitochondrial abnormalities and oxidative damage are major contributing factors for late-onset AD. To stop or delay the progression of late-onset AD, and also

to reduce disease symptoms, several therapeutic strategies have been developed, including anti-inflammatory, antioxidant, and anti-amyloid approaches. Among these, mitochondrial antioxidant therapy reduces AD pathology more than any other approach. However, until recently, a major limitation in developing antioxidant therapies for AD patients has been the inability to enhance antioxidant levels in mitochondria. There has been a breakthrough in the mitochondrial targeting of antioxidants. Mitochondrially targeted antioxidants have been developed, which preferentially enter the mitochondria—at several hundred-fold more than they enter natural antioxidants—where they rapidly neutralize free radicals and decrease mitochondrial toxicity. However, further research is needed to determine whether these mitochondrially targeted antioxidants can be used in mouse models of aging and in age-related neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's.

ABBREVIATIONS

3NP	3-nitropropionic acid
ABAD	alcohol dehydrogenase
A β	amyloid beta
ACh	acetylcholine
AD	Alzheimer's disease
ApoE	apolipoprotein E
APP	amyloid precursor protein
ATP	adenosine triphosphate
BChE	butylcholinesterase
ChAT	choline acetyltransferase
ETC	electron transport chain
H ₂ O ₂	hydrogen peroxide
MCAT	mitochondrial catalase
MitoVitE	mitochondrially targeted vitamin E
MitoQ	mitochondrially targeted ubiquinone
MitoPBN	mitochondrially targeted α -phenyl-N- <i>tert</i> -butyl nitron
N2a	neuroblastoma
NCAT	nuclear catalase
NFT	neurofibrillary tangle
O ₂ ^{•−}	superoxide anion
PBN	α -phenyl-N- <i>tert</i> -butyl nitron
ROS	reactive oxygen species
SOD	superoxide dismutase
tBHP	t-butylhydroperoxide

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