The AAPS Journal 2006; 8 (3) Article 62 (http://www.aapsj.org).

Themed Issue: NIDA/AAPS Symposium on Drugs of Abuse: Mechanisms of Toxicity, Toxicokinetics and Medical Consequences, November 4-5, 2005
Guest Editors - Rao S. Rapaka and Jagitsing H. Khalsa

Mitochondria-Targeted Peptide Antioxidants: Novel Neuroprotective Agents

Submitted: March 15, 2006; Accepted: April 21, 2006; Published: August 18, 2006

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Abstract

Increasing evidence suggests that mitochondrial dysfunction and oxidative stress play a crucial role in the majority of neurodegenerative diseases. Mitochondria are a major source of intracellular reactive oxygen species (ROS) and are particularly vulnerable to oxidative stress. Oxidative damage to mitochondria has been shown to impair mitochondrial function and lead to cell death via apoptosis and necrosis. Because dysfunctional mitochondria will produce more ROS, a feed-forward loop is set up whereby ROS-mediated oxidative damage to mitochondria favors more ROS generation, resulting in a vicious cycle. It is now appreciated that reduction of mitochondrial oxidative stress may prevent or slow down the progression of these neurodegenerative disorders. However, if mitochondria are the major source of intracellular ROS and mitochondria are most vulnerable to oxidative damage, then it would be ideal to deliver the antioxidant therapy to mitochondria. This review will summarize the development of a novel class of mitochondria-targeted antioxidants that can protect mitochondria against oxidative stress and prevent neuronal cell death in animal models of stroke, Parkinson's disease, and amyotrophic lateral sclerosis.

Keywords: Reactive oxygen species, mitochondrial permeability transition, apoptosis, necrosis, Parkinson's disease, amyotrophic lateral sclerosis

Introduction

Genetic studies have identified several genetic mutations that are associated with neurodegenerative diseases, including Parkinson's disease (PD), Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), Huntington's disease (HD), and Friedrich's ataxia (FRDA). It should be noted, however, that familial cases account for only a small percentage of these disorders; most cases of neurodegenerative disease are sporadic. Increasing evidence suggests that mitochondrial dysfunction and oxidative stress play a crucial role in the majority of neurodegenerative diseases. Mitochondria are a major source of intracellular reactive oxygen species (ROS) and are particularly vulnerable to oxidative stress. Oxidative damage to mitochondria has been shown to impair mitochondrial function and lead to cell death via apoptosis and necrosis. Because dysfunctional mitochondria will produce more ROS, a feed-forward loop is set up whereby ROS-mediated oxidative damage to mitochondria favors more ROS generation, resulting in a vicious cycle. It is now appreciated that reduction of mitochondrial oxidative stress may prevent or slow down the progression of these neurodegenerative disorders. This review will summarize the development of a novel class of mitochondria-targeted antioxidants that can protect mitochondria against oxidative stress and prevent neuronal cell death in animal models of stroke, PD, and ALS.

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Mitochondrial Dysfunction and Oxidative Damage in Neurodegenerative Diseases

A recent review by Beal[1] presented extensive evidence for the involvement of mitochondria in neurodegenerative diseases. Decreased mitochondrial complex I activity has been reported in the substantia nigra of postmortem brain samples from patients with PD, HD, and FRDA.[2] Inhibitors of complex I of the mitochondrial electron transport chain (ETC) produce PD in humans and animals, and knockouts of parkin or DJ-1, which are associated with familial PD, show defective mitochondrial function.[3,4] Alzheimer's brains harbor somatic mitochondrial DNA (mtDNA) mutations that suppress mitochondrial transcription and replication,[5] and there is evidence that β-amyloid is targeted to mitochondria, where it has been shown to bind ABAD (β-amyloid binding alcohol dehydrogenase) and to inhibit cytochrome c oxidase.[6,7] Mice overexpressing mutant CuZn superoxide dismutase (SOD1), a model for ALS, showed compromised mitochondrial respiration and adenosine triphosphate (ATP) production.[8,9] Reduced expression of frataxin in a model of FRDA leads to iron accumulation in mitochondria and impairment of complex I and II of the ETC.[10] Finally, impaired mitochondrial function has been shown in patients with HD, as well as animal models of HD (see review[11]).

Mitochondrial dysfunction, whether caused by genetic mutations or environmental factors, leads to increased production of ROS. Evidence that oxidative stress plays a role in PD, AD, and ALS is accumulating.[12-14] Postmortem analyses have revealed that overall levels of oxidative damage to proteins, lipids, and DNA are elevated in AD and PD brains.[15] Oxidative changes to proteins such as α-synuclein in PD, β-amyloid in AD, and SOD in ALS might result in protein misfolding and aggregate formation.[14] A direct association between ROS production and amyloid plaques has also been demonstrated in transgenic mice and in human AD brain tissue.[16] Several oxidative biomarkers are also elevated in a mouse model of ALS.[17] The brain may be particularly susceptible to oxidative stress because of its high metabolic rate and reduced capacity for cellular regeneration.

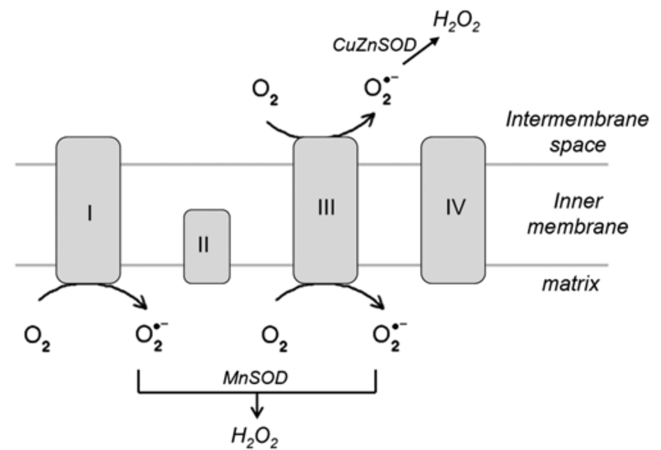

Figure 2. Mitochondrial superoxide production and disposition. SOD indicates superoxide dismutase.

Mitochondrial Generation of ROS

Mitochondria consume nearly 85% to 90% of a cell's oxygen to support oxidative phosphorylation by harnessing oxidized fuel to the synthesis of ATP (Figure 1). The energy released by the flow of electrons through the ETC is used to pump protons out of the mitochondria inner membrane through complexes I, III, and IV. This creates an electrochemical gradient across the mitochondrial inner membrane. The potential energy stored is coupled to ATP synthesis by complex V (ATP synthase). Oxygen normally serves as the ultimate electron acceptor and is reduced to water. However, electron leak to oxygen through complexes I and III can generate superoxide anion ($O_2^{\bullet-}$) (Figure 2).[18] The rate of $O_2^{\bullet-}$ production is affected by mitochondrial metabolic state and increases when the electron carriers harbor excess electrons, either from inhibition of oxidative phosphorylation or from excessive calorie consumption. The location of $O_2^{\bullet-}$ within mitochondria is important because $O_2^{\bullet-}$ does not diffuse across membranes. Recent studies suggest that complex I releases $O_2^{\bullet-}$ into the matrix while complex III can release $O_2^{\bullet-}$ into the matrix as well as the intermembrane space (Figure 2).[19] Superoxide anion can be converted to H_2O_2 by mitochondrial matrix enzyme MnSOD or by CuZnSOD in the intermembrane space. H_2O_2 is more stable than $O_2^{\bullet-}$ and can diffuse out of the mitochondrion and into the cytosol (Figure 3). However, in the presence of reduced transitional metals, H_2O_2 can be converted to the highly reactive hydroxyl radical ($OH^\bullet$) via the Fenton reaction (Figure 3). $OH^\bullet$ may further react with bicarbonate to yield the very reactive carbonate radical anion ($CO_3^{\bullet-}$). $O_2^{\bullet-}$ can also react with another free radical, nitric oxide ($NO^\bullet$), formed by mitochondrial nitric oxide synthase, to generate the highly reactive peroxynitrite ($ONOO^-$) (Figure 3).

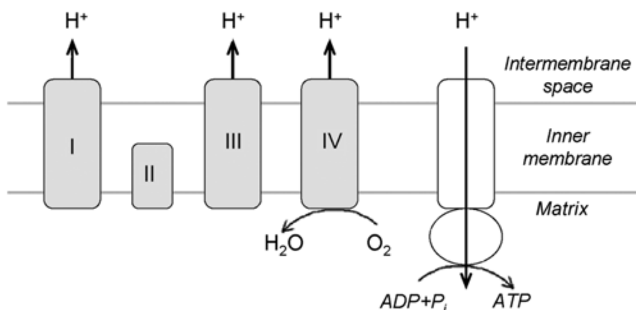

Figure 1. The electron transport chain and ATP synthesis on the mitochondria inner membrane. ATP indicates adenosine triphosphate; ADP, adenosine diphosphate.

Free Radical Damage to Mitochondria

Although H_2O_2 may freely diffuse out of mitochondria, other radicals such as $O_2^{\bullet-}$ and $OH^\bullet$ are largely limited by diffusion and likely to inflict damage directly to mitochondria, especially to the inner membrane where they are

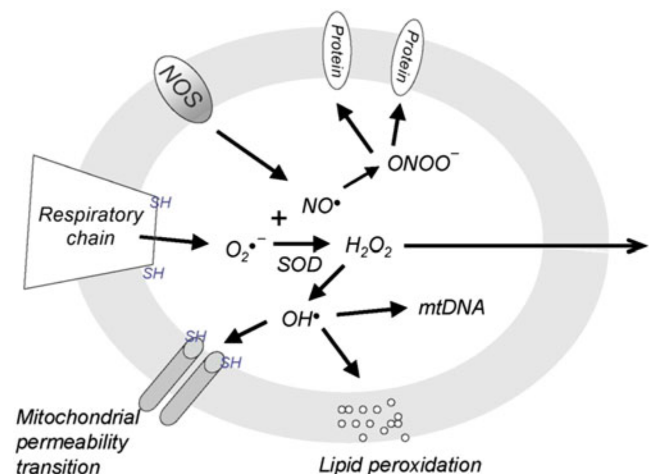

Figure 3. Mitochondrial damage caused by reactive oxygen and nitrogen species. NOS indicates nitric oxide synthase; SH, thiol groups; SOD, superoxide dismutase; mtDNA, mitochondrial DNA.

generated. Furthermore, the inner membrane contains many iron and copper complexes, which may catalyze further reactions between $O_2^{\bullet -}$ and H_2O_2. ROS can initiate damage to nucleic acids, proteins, and lipids (Figure 3).[20] Mitochondrial DNA are more vulnerable than nuclear DNA to oxidative damage because they are situated much closer to the site of ROS generation, and mitochondria lack protective histones and have much more limited base excision repair mechanisms than they do nuclear repair mechanisms.[21] Mitochondrial proteins can be modified by nitration, S-nitrosylation, S-glutathionylation, or carbonylation. Protein oxidation and nitration result in altered function of many metabolic enzymes in the mitochondrial ETC, including nicotinamide adenine dinucleotide (NADH) dehydrogenase, NADH oxidase, cytochrome c oxidase, and ATPase.[20,22] Oxidation of the adenine nucleotide translocator (ANT) will impair the influx of adenosine diphosphate (ADP) into the matrix for ATP synthesis. Another particularly relevant protein that loses function upon oxidation is MnSOD; its loss of function would further compromise antioxidant capacity and lead to further oxidative stress.[23] ROS, especially $OH^{\bullet}$, can lead directly to peroxidation of lipids and the production of highly reactive aldehyde species such as 4-hydroxy-2,3,trans-nonenal, which can then result in secondary detrimental effects.[24] Cardiolipin, the major phospholipid on the mitochondria inner membrane, is particularly susceptible to peroxidation because of its high degree of unsaturation.[25]

Mitochondrial Antioxidant Network

Mitochondria are normally protected from oxidative damage by a multilayer network of mitochondrial antioxidant systems (see review[26]). H_2O_2 can be readily converted to water by mitochondrial glutathione peroxidase, which oxidizes reduced glutathione (GSH) to oxidized glutathione (GSSG). Glutathione reductase then converts GSSG back to GSH. GSH is synthesized in the cytosol and must be imported into mitochondria by a transporter. In addition to GSH, mitochondria have 2 other small thiol-disulfide oxidoreductases—thioredoxin and glutaredoxin—that play important roles in thiol redox control. Another detoxification enzyme, catalase, is present only in heart mitochondria. In addition to these antioxidant enzymes, mitochondria possess several low-molecular-weight antioxidants, including α-tocopherol and ubiquinol. These molecules are particularly effective in scavenging lipid peroxyl radicals and preventing the free radical chain reaction of lipid peroxidation.

Mitochondrial Oxidative Damage and Cytochrome C Release

Mitochondria undergo oxidative damage when ROS production exceeds the antioxidant capacity of mitochondria. One outcome of mitochondrial oxidation is the release of mitochondrial cytochrome c into the cytosol. Cytochrome c is normally bound to the inner mitochondrial membrane by an association with cardiolipin.[27] Peroxidation of cardiolipin leads to dissociation of cytochrome c and its release through the outer mitochondrial membrane.[28] The mechanism by which cytochrome c is released through the outer membrane is not clear. One mechanism may involve mitochondrial permeability transition (MPT). The MPT pore is a high conductance channel that is believed to be formed by the apposition of the voltage-dependent anion channel on the outer membrane and the ANT on the inner membrane.[29] The opening of the MPT pore causes a sudden increase in the permeability of the inner mitochondrial membrane. This results in swelling of the mitochondrial matrix, rupture of the outer membrane, and release of cytochrome c.[30] ROS may promote MPT by causing oxidation of thiol groups on the ANT.[31] Cytochrome c release may also occur via MPT-independent mechanisms and may involve an oligomeric form of Bax.[32] Bax and MPT may also work together to cause cytochrome c release.[33]

Mitochondrial Oxidative Damage and Cell Death

Cytochrome c released from mitochondria into the cytoplasm binds to Apaf-1 to initiate the formation of an apoptosome, which then binds pro-caspase-9. The oligomerization of caspase-9 on the apoptosome activates the protease. The active caspase-9 cleaves 2 "executioner" caspases, caspase-3 and caspase-7, that then go on to cleave key substrates within the cell.[34,35] This has been termed the intrinsic mitochondrial pathway of apoptosis. Depending on the availability of intracellular ATP, the cell death pathway may switch from apoptosis to necrosis (Figure 4). Several enzymes of the ETC have been shown to be inhibited by ROS, and opening of the MPT pore results in mitochondrial depolarization and inhibition of ATP synthesis. In addition, single-strand DNA breaks mediated by ROS activate poly-ADP-ribose polymerase (PARP) for DNA repair. Activated PARP cleaves NAD^+ into nicotinamide and ADP-ribose, resulting in NAD^+ and ATP depletion.[36] While apoptosis is the predominant cell death pathway in the presence of adequate ATP, overwhelming depletion of ATP results in necrotic cell death.

Antioxidants as Therapy for Neurodegenerative Diseases

There is evidence that some antioxidant treatments can attenuate or delay disease progression in animal models of neurodegenerative diseases. Natural antioxidants ranging from coenzyme Q (CoQ), vitamin E (tocopherol), and

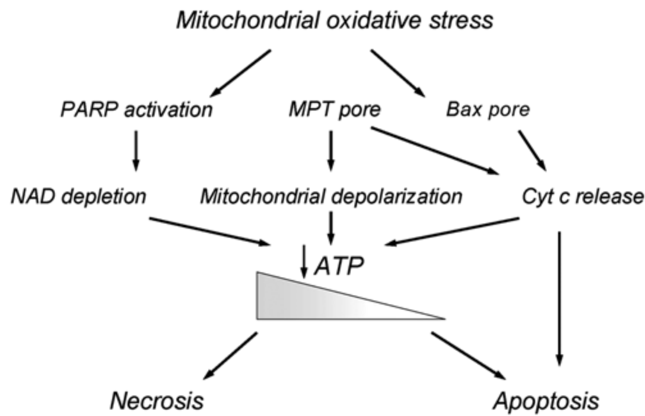

Figure 4. Mitochondrial oxidative damage leading to apoptosis or necrosis is dependent on availability of ATP. PARP indicates poly-ADP-ribose-polymerase; ADP, adenosine diphosphate; MPT, mitochondrial permeability transition; NAD, nicotinamide adenine dinucleotide; Cyt, cytochrome; ATP, adenosine triphosphate.

creatine to green tea polyphenols have shown protective effects in mice models of PD, ALS, and AD.[37,38] However, clinical trials with the same antioxidants have failed to show benefits in humans, and high doses of vitamin E may actually be harmful.[39] The difficulty many antioxidants have with penetrating the blood-brain barrier (BBB) is one possible reason for their lack of efficacy. It has been reported that feeding rats with CoQ for 2 months failed to increase brain CoQ levels.[40] Furthermore, some antioxidants may not reach the relevant sites of free radical generation (Figure 5). Large proteins like SOD and catalase do not penetrate cell membranes and are therefore ineffective against intracellular ROS. Vitamin E and CoQ are very lipophilic, tend to be retained in cell membranes, and fail to achieve significant intracellular concentrations. To overcome the limitations of natural antioxidants, several low-molecular-weight catalytic antioxidants, generally referred to as SOD mimetics, have been developed (see review[41]). Several of these SOD mimetics have been reported to be effective in blocking oxidant stress in cell models, including offering protection against cell death induced by 1-methyl-4-phenylpyridium (MPP^+) and paraquat, models of PD.[42,43] Two SOD mimetics (EUK-8 and EUK-134) were shown to prolong survival in an ALS model (SOD1 transgenic mice),[44] and systemic administration of EUK-189 reduced striatal dopaminergic cell death caused by paraquat[45] A manganese porphyrin (AEOL 10150) administered to G93A SOD1 mutants (ALS model) at the onset of symptoms prolonged survival by 11%.[46] However, another SOD mimetic, MnTBAP, failed to provide neuroprotection in a SOD1 knockout mouse because it did not cross the BBB.[47] If mitochondria are the major source of intracellular ROS and mitochondria are most vulnerable to oxidative damage, then it would be ideal to deliver the antioxidant therapy to mitochondria. Recently it was shown that overexpression of catalase in mitochondria increased life span by 20% in mice, whereas overexpression of catalase in peroxisomes had no significant effect.[48] Thus, antioxidant efficacy may be determined by targeted delivery to the site of ROS production.

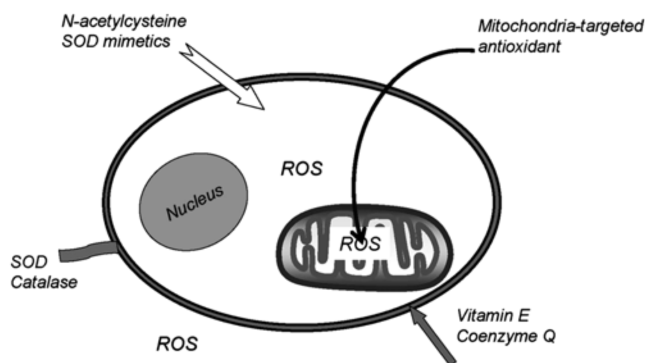

Figure 5. Compartmentalization of ROS and cellular delivery of antioxidants. SOD indicates superoxide dismutase; ROS, reactive oxygen species.

Mitochondria-Targeted Antioxidants—Mitochondrial Potential-Dependent Delivery

TPP^+-Conjugated Vitamin E and CoQ

The development of mitochondria-targeted antioxidants was recently reviewed by Sheu et al.[49] The most common method for targeting compounds to mitochondria makes use of the potential gradient across the mitochondrial inner membrane. As a result of the proton gradient, a negative potential of 150 to 180 mV is generated across the inner membrane (Figure 1). Lipophilic cations may therefore accumulate 100- to 1000-fold in the mitochondrial matrix. Murphy and coworkers applied this approach by conjugating the triphenylphosphonium cation (TPP^+) to lipophilic antioxidants such as CoQ (MitoQ) and vitamin E (MitoVitE).[50] MitoQ and MitoVitE are both taken up into the mitochondrial matrix in a potential-dependent manner and are effluxed out of mitochondria upon depolarization. By preferentially accumulating in the mitochondrial matrix, MitoVitE was reported to be 800-fold more potent than idebenone in protecting against GSH depletion in cultured fibroblasts from patients with FRDA, and MitoVitE was 350-fold more potent than trolox (water-soluble vitamin E analog).[51] This enhanced potency of the TPP^+-conjugated antioxidants was abolished in cells pretreated with FCCP (carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone) to depolarize mitochondria. MitoQ and MitoVitE were also reported to inhibit H_2O_2-induced apoptosis in endothelial cells at concentrations of 1 μM.[52]

In vivo distribution of the [^{3}H]TPMP (methyltriphenylphosphonium) cation alone was studied in mice after

intraperitoneal (IP) and intravenous (IV) administration.[53] Uptake of [^{3}H]TPMP was observed in the liver 1 hour after IP injection, but none was detected in the brain, and for unknown reasons, detectable levels were found in the heart only after 20 hours. When injected IV, [^{3}H]TPMP was distributed to all tissues. The authors mentioned that the distribution of [^{3}H]MitoVitE 4 hours after IP administration was similar to that of [^{3}H]TPMP, but data were not provided. Surprisingly, when [^{3}H]MitoQ and [^{3}H]MitoVitE were given in drinking water to mice, detectable levels were found in the liver and the heart after 4 days, although levels in brain tissue remained lower than levels in all other tissues. Isolated hearts taken from rats treated with MitoQ in their drinking water for 14 days were found to be protected ex vivo against ischemia-reperfusion injury.[54] These data suggest that distribution of the TPP^+-conjugated antioxidants to the brain is quite restricted and their usefulness for neuroprotection is questionable. These TPP^+-conjugated antioxidants have not been evaluated in vivo in animal models of neurodegenerative diseases.

The utility of TPP^+-conjugated antioxidants may also be limited by their requirement of mitochondrial potential for mitochondrial uptake. All the neurodegenerative diseases are associated with impaired mitochondrial function and are unlikely to have normal mitochondrial potential. Furthermore, the uptake of these antioxidants is self-limiting in that uptake is reduced at concentrations greater than 50 μM.[55] This may be due to the inevitable depolarization of mitochondria when large amounts of cations accumulate in the matrix. In fact, high concentrations of MitoQ have been shown to cause mitochondrial depolarization,[55,56] and this may be a problem with chronic use. In addition, MitoQ can undergo autoxidation in mitochondria to generate $O_2^{\bullet -}$ and causes H_2O_2 efflux from mitochondria. Furthermore, MitoQ does not prevent peroxide-induced thiol oxidation and therefore does not prevent MPT pore opening caused by thiol oxidation.[57]

Choline Esters of Glutathione and N-Acetyl-Cysteine

Glutathione (L-γ-glutamyl-L-cysteinyl-glycine) plays a very important role in detoxifying ROS and preventing thiol oxidation. Glutathione is synthesized in the cytoplasm and transported into mitochondria via specific carriers. Increasing mitochondrial glutathione can be very effective in preventing mitochondrial oxidative stress. N-acetyl-L-cysteine is often used to provide cysteine for glutathione synthesis. Using a similar approach as with the TPP^+ conjugated antioxidants, Sheu et al prepared choline esters of glutathione and N-acetyl-cysteine (NAC) to enhance the uptake of glutathione and NAC into mitochondria.[49] Early studies have shown that glutathione and NAC can protect against oxidative stress in cultured cells, but in vivo animal studies are not yet available.

MITOCHONDRIA-TARGETED ANTIOXIDANTS—MITOCHONDRIAL POTENTIAL-INDEPENDENT DELIVERY

A novel class of small cell–permeable peptide antioxidants that target mitochondria in a potential-independent manner was reported recently.[58] The structural motif of these Szeto-Schiller (SS) peptides centers on alternating aromatic residues and basic amino acids (aromatic-cationic peptides).[58,59] The tyrosine (Tyr)- or dimethyltyrosine (Dmt)-containing analogs can scavenge H_2O_2, $OH^{\bullet}$, and $ONOO^{\bullet}$ and inhibit lipid peroxidation in vitro, as demonstrated by the inhibition of linoleic acid oxidation and low-density lipoprotein (LDL) oxidation. Tyrosine can scavenge oxyradicals, forming relatively unreactive tyrosyl radicals, which can be followed by radical-radical coupling to give dityrosine or can react with superoxide to form tyrosine hydroperoxide.[60] Dimethyltyrosine is more effective than tyrosine in scavenging of ROS. The specific location of the tyrosine or dimethyltyrosine residue did not seem to be important, as SS-31 (D-Arg-Dmt-Lys-Phe-NH_2) was found to be as effective as SS-02 (Dmt-D-Arg-Phe-Lys-NH_2) in scavenging H_2O_2 and inhibiting LDL oxidation. Replacement of tyrosine with phenylalanine (SS-20; Phe-D-Arg-Phe-Lys-NH_2) eliminated all scavenging ability.[58]

These small peptides contain an amino acid sequence that allows them to freely penetrate cells despite carrying a 3+ net charge at physiologic pH.[61] These aromatic-cationic peptides are taken up into cells in an energy-independent, nonsaturable manner. Uptake studies with [^{3}H]SS-02 and [^{3}H]SS-31 showed rapid cellular uptake, with steady state achieved in less than 30 minutes.[61,62] This suggests that these peptides can freely pass through the plasma membrane in both directions. Unlike the larger cationic peptides like *Tat* peptide, the SS peptides show no evidence of vesicular localization that would result from endocytosis.[63,64]

These SS peptides have a sequence motif that makes them targeted to mitochondria. Confocal microscopy showed rapid uptake of a fluorescent analog of SS-02 (SS-19; Dmt-D-Arg-Phe-atnDap-NH_2; atn = β-anthraniloyl-L-α,β-diaminopropionic acid)[65] in living cells.[58] The intracellular distribution pattern of the fluorescent peptide analog was identical to that of Mitotracker TMRM, a fluorescent dye that is taken up into mitochondria in a potential-driven manner, suggesting that these peptides are targeted to mitochondria.[58] Incubation of isolated mouse liver or brain mitochondria with [^{3}H]SS-02 or [^{3}H]SS-31 confirmed that these peptides are taken up and concentrated 1000-fold and 10 000-fold, respectively, in mitochondria.[58,62] Contrary to the mitochondrial uptake of MitoQ and MitoE, the uptake of these aromatic-cationic peptides into mitochondria is not dependent on mitochondrial potential, as the extent of uptake was reduced by only ~10% to 15% in mitochondria

that were depolarized with FCCP.[58] Mitochondria fractionation studies showed that ~85% of the [^{3}H]SS-02 was associated with the inner membrane, further suggesting that these peptides are not targeted to the mitochondrial matrix via potential-dependent mechanisms. Because these positively charged peptides are not delivered into the mitochondrial matrix, their uptake is not self-limiting, and they do not cause mitochondrial depolarization even at 1 mM.[58]

Mitochondria-Targeted Peptide Antioxidants Inhibit Mitochondrial ROS Production and Prevent Mitochondrial Swelling

By targeting the inner membrane, the SS peptides reduce mitochondrial ROS accumulation in a dose-dependent manner. The Dmt-containing peptide analogs, SS-02 and SS-31, inhibited H_2O_2 release when incubated with isolated mouse liver mitochondria at 37°C (Figure 6A). Mitochondrial ROS production can be prevented by mitochondrial depolarization with the use of uncouplers, but none of the SS peptides decreased mitochondrial potential in isolated mitochondria as measured with Mitotracker TMRM (Figure 6B). Although it lacks scavenging ability, SS-20 also significantly decreased H_2O_2 release, but the response was dramatically less compared with the response with the scavenging peptide antioxidants SS-02 and SS-31. Mitochondria ROS generation is known to contribute to mitochondrial swelling caused by high calcium and inorganic phosphate, conditions that mimic ischemia. By reducing mitochondrial ROS production, SS-31 inhibited mitochondrial swelling induced by calcium and P_i in a dose-dependent manner and thereby prevented cytochrome c release from mitochondria.[58] Mitochondrial calcium overload is known to stimulate ROS production, and the scavenging peptides may protect mitochondria by scavenging ROS. SS-20, which cannot scavenge H_2O_2, was unable to prevent calcium-induced mitochondrial swelling.[58] Furthermore, although trolox can inhibit mitochondrial H_2O_2 accumulation, it was unable to prevent calcium-induced mitochondrial swelling (Figure 6C). This is likely because trolox does not penetrate into mitochondria and can scavenge only extramitochondrial H_2O_2. Thus, it appears that targeted delivery of antioxidants to the mitochondrial inner membrane is necessary to protect against Ca^{2+}-mediated mitochondrial swelling.

SS Peptides Protect Mitochondria Against Oxidative Damage in Cultured Cells

By targeting and concentrating >1000-fold in the mitochondrial inner membrane, these SS peptides are extremely potent in preventing oxidative cell death. *Tert*-butylhydroperoxide (*t*BHP) is a membrane-permeant prooxidant compound that can induce cell death via apoptosis or necrosis.[66,67] *t*BHP can generate *t*-butoxyl radicals via the Fenton reaction resulting in lipid peroxidation, depletion of intracellular glutathione, modification of protein thiols, depletion of intracellular ATP stores, and loss of cell viability. Treatment of cells with *t*BHP causes rapid oxidation of pyridine nucleotides and increased ROS production in mitochondria.[68,69] SS-31 was very potent in reducing intracellular ROS and preventing cell death after *t*BHP treatment, with EC_{50} in the nM range.[58,62] In contrast, most antioxidants require at least 100 μM to reduce oxidative cell death.[51,69,70] MitoQ was able to block H_2O_2-induced apoptosis at 1 μM, but >10 μM caused cytotoxicity.[56]

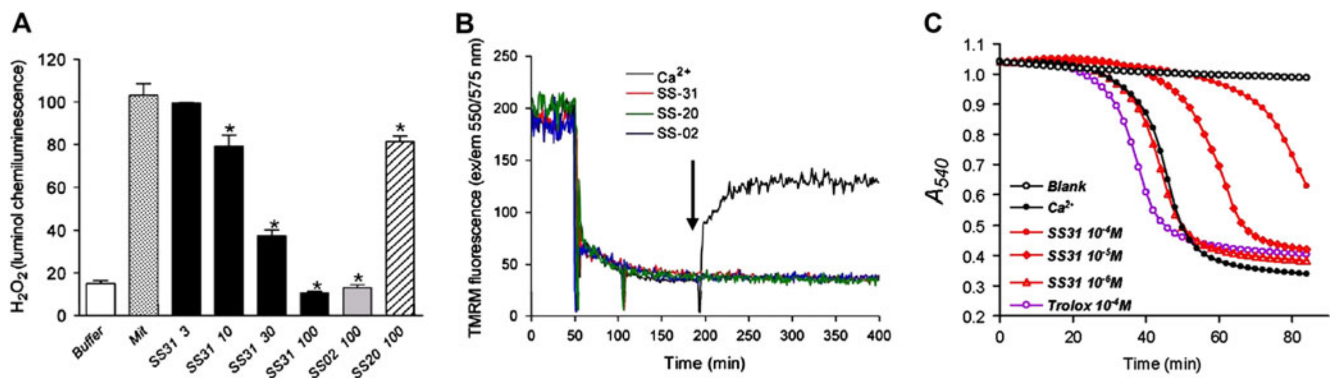

Figure 6. SS peptides reduce mitochondrial H_2O_2 accumulation and prevent Ca^{2+}-induced mitochondrial swelling. (A) Isolated mouse liver mitochondria were incubated at 37°C, and H_2O_2 was measured by luminol luminescence. SS-31 and SS-02 reduced H_2O_2 accumulation in a dose-dependent manner. SS-20, the nonscavenging analog, was much less potent in reducing H_2O_2 accumulation. (B) The reduction in H_2O_2 accumulation by SS peptides was not due to mitochondrial depolarization. Mitochondrial potential was measured using Mitotracker TMRM. Addition of Ca^{2+} (indicated by arrow) caused immediate depolarization, while addition of SS-02, SS-31, and SS-31 did not alter mitochondrial potential. (C) SS-31 inhibited Ca^{2+}-induced mitochondrial swelling in a dose-dependent manner. Isolated mouse liver mitochondria were exposed to 50 μM Ca^{2+}, and swelling was measured by absorbance at 540 nm. Addition of 100 μM of trolox had no effect on mitochondrial swelling. SS indicates Szeto-Schiller peptides.

In contrast, no toxicity was observed with 1 mM SS-02 or SS-31.

Recent evidence suggests that *t*BHP-induced apoptosis is triggered by MPT.[67] The scavenging SS peptides (SS-02 and SS-31) were able to inhibit MPT, prevent mitochondrial swelling, and reduce cytochrome c release in response to Ca^{2+} overload.[58] In neuroblastoma N_2A cells exposed to *t*BHP, SS-31 prevented mitochondrial depolarization at subnanomolar concentrations and inhibited apoptosis.[62] These results support the proposal that ROS may potentiate MPT via oxidation of the adenine nucleotide translocator. The ability of SS peptides to prevent MPT will minimize MPT-induced ROS accumulation and reduce further oxidative damage on mitochondria.

Pharmacokinetic Properties of SS Peptides

The SS peptide antioxidants are highly "druggable" and have excellent pharmacokinetic profiles. They are small and easy to synthesize, readily soluble in water, and resistant to peptidase degradation. The presence of a *D*-amino acid in either the first or the second position minimizes aminopeptidase degradation, and amidation of the C-terminus reduces hydrolysis from the C-terminus. In addition to cellular uptake, transcellular uptake probably occurs: SS-02 was shown to readily penetrate a monolayer of intestinal epithelial cells in both apical-basolateral and basolateral-apical directions.[61] Since these cells possess tight junctions, the results suggest that SS-02 undergoes transcellular uptake. Most important in the consideration of drug development for neuroprotection is whether the drugs can penetrate the BBB. Pharmacokinetic studies have revealed rapid distribution and relatively long elimination half-life for SS-02 in sheep[71] and rats (H.H. Szeto and D.M. Desiderio, unpublished data, 2005). Studies have shown that [^{3}H]SS-02 can be detected in mouse brain within 5 minutes after IV injection (A. Gifford, unpublished data, 2004). This ability of SS-02 to penetrate the BBB is confirmed by the observation that SS-02, which also possess high affinity for the μ opioid receptor, is a very potent analgesic after subcutaneous (SC) administration in mice.[72] The duration of analgesia achieved with a single SC dose of SS-02 is 4 times longer than the duration of analgesia with an equipotent dose of morphine.[65]

SS Peptides Protect Against Ischemia-Reperfusion Injury Ex Vivo and In Vivo

Mitochondrial permeability transition and ROS are thought to play a major role in ischemia-reperfusion injury.[73] ROS production during reperfusion is believed to be the triggering event in ischemia-reperfusion injury. Pretreatment with M40403, a small-molecule SOD mimetic, 30 minutes before ligation of the left anterior descending coronary artery significantly reduced oxidative damage to the rat heart, reduced neutrophil infiltration, and decreased infarct size.[74] Pretreatment of rats with MitoQ in drinking water for 14 days improved cardiac function in the ex vivo heart subjected to acute ischemia-reperfusion,[54] but the efficacy of MitoQ has not been investigated in vivo. The efficacy of free radical scavengers in animal studies is often not observed in clinical trials, and this may at least in part be attributed to their administration prior to ischemia in most animal studies, rather than upon reperfusion, as is usually done in patients with acute myocardial infarction. Postischemic administration of a metalloporphyrin catalytic antioxidant (AEOL10113) significantly reduced infarct size in rats subjected to 90-minute occlusion of the middle cerebral artery, but that compound had to be given intracerebroventricularly.[75]

Reperfusion of the ex vivo heart after short-term ischemia is associated with progressive decline in cardiac contractile force despite recovery of coronary flow and heart rate. This phenomenon, termed myocardial stunning, is also observed in clinical patients and is thought to be mediated by ROS generation during reperfusion. Both SS-02 and SS-31 were able to prevent myocardial stunning when administered upon reperfusion after 30-minute ischemia in the ex vivo guinea pig heart.[58,76] In contrast, SS-20, which has no scavenging ability, was unable to prevent myocardial stunning when administered after ischemia. The ability of SS-02 to prevent myocardial stunning has been confirmed in rats in vivo,[77] and recent studies have shown that pre-ischemic administration of SS-31 IP to rats significantly reduced infarct size.[78] These findings with the SS peptides support the proposal that ROS play a major role in reperfusion-induced myocardial stunning.

SS-31 has also been reported to reduce infarct volume when administered to mice after acute cerebral ischemia.[79] Mice were subjected to 30-minute unilateral occlusion of the middle cerebral artery. SS-31 (2 mg/kg) was administered to mice shortly after onset of reperfusion. Animals treated with SS-31 showed significantly reduced GSH depletion as well as infarct volume and hemispheric swelling compared with saline-treated animals. These data suggest that SS-31 treatment upon reperfusion can minimize ischemic damage by maintaining GSH levels in the ischemic brain.

SS Peptides Prevent Neuronal Cell Loss in Animal Models of Neurodegeneration

SS-31 has been evaluated in animal models of 2 neurodegenerative diseases, PD and ALS.

MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) is a neurotoxin that produces in humans an irreversible and severe parkinsonian syndrome characterized by all the features of idiopathic PD. MPTP is converted by astrocytes to

MPP^+, which is then taken up into the dopaminergic neurons via the dopamine transporter. Within dopamine neurons, MPP^+ inhibits mitochondrial complex-I activity and leads to neuronal cell death. Treatment of mice with SS-31 in a dose-dependent manner (0.5-2 mg/kg, SC) prevented the loss of striatal dopamine neurons caused by MPTP and preserved striatal dopamine levels (L. Yang and F. Beal, unpublished data, 2005). Studies with isolated brain mitochondria and dopamine neurons in culture confirmed the protection offered by SS-31 against MPP^+ exposure (K. Zhao and H.H. Szeto, unpublished data, 2005).

Mutations in CuZn SOD1 have been found in 10% to 20% of ALS patients. Overexpression of G93A SOD1 in mice is a common animal model for ALS. Mutant SOD1 is recruited to spinal cord mitochondria, where it can form aggregates and cause mitochondrial dysfunction.[80] In one study, daily injections of SS-31 (5 mg/kg, SC) to G93A SOD1 mutants before onset of symptoms led to a significant increase in survival and improvement of motor performance.[81] The gain in motor performance was associated with significantly reduced cell loss in the lumbar spinal cord and a reduction in the level of several oxidative markers.

These studies support the assertion that mitochondrial dysfunction and oxidative damage are major contributors to the pathophysiology of PD and ALS, and that mitochondria-targeted antioxidants such as SS-31 represent a novel therapeutic approach to these and other neurodegenerative disorders.

CONCLUSION

Oxidative damage is believed to be associated with aging and numerous degenerative diseases. However, available antioxidants have not proven to be particularly effective against many of these disorders. A possibility is that some of the antioxidants do not reach the relevant sites of free radical generation, especially if mitochondria are the primary source of ROS generation. The SS antioxidant peptides represent a novel approach, with targeted delivery of antioxidants to the mitochondrial inner membrane, the site of ROS production. By protecting mitochondrial viability, these peptides can also minimize further ROS generation, thus breaking the vicious cycle between mitochondrial dysfunction and ROS (Figure 7). By selectively concentrating in mitochondria, these peptides are extraordinarily potent in protecting against oxidative cell death. Preclinical studies support their potential use for ischemia-reperfusion injury and neurodegenerative disorders. Although peptides have often been considered poor drug candidates, these small peptides have excellent "druggable" properties, and their ease in crossing the BBB makes them promising agents for the many neurodegenerative diseases for which better treatment strategies are needed.

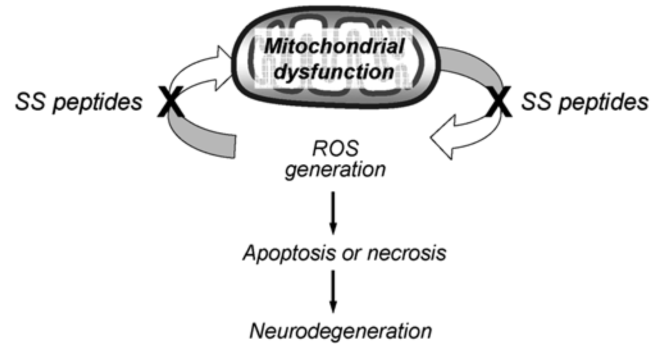

Figure 7. SS peptide antioxidants break the vicious cycle between ROS and mitochondrial dysfunction. SS indicates Szeto-Schiller; ROS, reactive oxygen species.

ACKNOWLEDGMENTS

I am grateful to the many collaborators who contributed to the work presented in this review. The majority of the work was supported by the National Institute on Drug Abuse (PO1 DA08924) and the National Institute of Neurological Disorders and Stroke (R21 NS48295). The SS peptides were developed in collaboration with Dr Peter W. Schiller of the Clinical Research Institute of Montreal, Montreal, Quebec, Canada. All biochemical and cellular work mentioned in this article was performed by Drs Kesheng Zhao, Guomin Zhao, Dunli Wu, and Yi Soong. Special thanks to Dr Dominic Desiderio (Charles B. Stout Mass Spectrometry Lab, University of Tennessee, Memphis, TN) and Dr Andrew Gifford (Brookhaven National Labs, Long Island, NY) for their contributions to the pharmacokinetic studies. Dr Mun Hong (Department of Medicine, Weill Medical College of Cornell University) performed the in vivo studies with SS-02 in myocardial ischemia-reperfusion, and Dr Sunghee Cho (Burke Research Institute, White Plains, NY) did the studies with SS-31 in cerebral ischemia-reperfusion. The neurodegenerative studies were performed by Drs Lichuan Yang, Suzanne Petri, and Flint Beal of the Department of Neurology and Neuroscience, Weill Medical College of Cornell University.

Financial Disclosure: Patent applications have been filed by Cornell Research Foundation Inc (CRF) for the technology (SS peptides) described in this article. Hazel H. Szeto is the inventor. CRF, on behalf of Cornell University, has licensed the technology for further research and development to a commercial enterprise in which CRF and Dr. Szeto have financial interests.

REFERENCES

1. Beal MF. Mitochondria take center stage in aging and neurodegeneration. *Ann Neurol*. 2005;58:495-505.

2. Schapira AH. Mitochondrial involvement in Parkinson's disease, Huntington's disease, hereditary spastic paraplegia and Friedreich's ataxia. *Biochim Biophys Acta*. 1999;1410:159-170.

3. Greene JC, Whitworth AJ, Kuo I, Andrews LA, Feany MB, Pallanck LJ. Mitochondrial pathology and apoptotic muscle degeneration in Drosophila parkin mutants. *Proc Natl Acad Sci USA*. 2003;100: 4078-4083.

4. Palacino JJ, Sagi D, Goldberg MS, et al. Mitochondrial dysfunction and oxidative damage in parkin-deficient mice. *J Biol Chem*. 2004;279:18614-18622.

5. Coskun PE, Beal MF, Wallace DC. Alzheimer's brains harbor somatic mtDNA control-region mutations that suppress mitochondrial transcription and replication. *Proc Natl Acad Sci USA*. 2004;101:10726-10731.

6. Lustbader JW, Cirilli M, Lin C, et al. ABAD directly links Abeta to mitochondrial toxicity in Alzheimer's disease. *Science*. 2004;304:448-452.

7. Crouch PJ, Blake R, Duce JA, et al. Copper-dependent inhibition of human cytochrome c oxidase by a dimeric conformer of amyloid-beta1-42. *J Neurosci*. 2005;25:672-679.

8. Beckman JS, Estevez AG, Crow JP, Barbeito L. Superoxide dismutase and the death of motoneurons in ALS. *Trends Neurosci*. 2001;24:S15-S20.

9. Mattiazzi M, D'Aurelio M, Gajewski CD, et al. Mutated human SOD1 causes dysfunction of oxidative phosphorylation in mitochondria of transgenic mice. *J Biol Chem*. 2002;277:29626-29633.

10. Ferreirinha F, Quattrini A, Pirozzi M, et al. Axonal degeneration in paraplegin-deficient mice is associated with abnormal mitochondria and impairment of axonal transport. *J Clin Invest*. 2004;113:231-242.

11. Browne SE, Beal MF. The energetics of Huntington's disease. *Neurochem Res*. 2004;29:531-546.

12. Beal MF. Oxidatively modified proteins in aging and disease. *Free Radic Biol Med*. 2002;32:797-803.

13. Reddy PH, Beal MF. Are mitochondria critical in the pathogenesis of Alzheimer's disease? *Brain Res Brain Res Rev*. 2005;49:618-632.

14. Andersen JK. Oxidative stress in neurodegeneration: cause or consequence? *Nat Med*. 2004;10:S18-S25.

15. Giasson BI, Ischiropoulos H, Lee VM, Trojanowski JQ. The relationship between oxidative/nitrative stress and pathological inclusions in Alzheimer's and Parkinson's diseases. *Free Radic Biol Med*. 2002;32:1264-1275.

16. McLellan ME, Kajdasz ST, Hyman BT, Bacskai BJ. In vivo imaging of reactive oxygen species specifically associated with thioflavine S-positive amyloid plaques by multiphoton microscopy. *J Neurosci*. 2003;23:2212-2217.

17. Casoni F, Basso M, Massignan T, et al. Protein nitration in a mouse model of familial amyotrophic lateral sclerosis: possible multifunctional role in the pathogenesis. *J Biol Chem*. 2005;280: 16295-16304.

18. Turrens JF. Superoxide production by the mitochondrial respiratory chain. *Biosci Rep*. 1997;17:3-8.

19. Muller FL, Liu Y, Van RH. Complex III releases superoxide to both sides of the inner mitochondrial membrane. *J Biol Chem*. 2004;279: 49064-49073.

20. Cadenas E, Davies KJ. Mitochondrial free radical generation, oxidative stress, and aging. *Free Radic Biol Med*. 2000;29: 222-230.

21. Imam SZ, Karahalil B, Hogue BA, Souza-Pinto NC, Bohr VA. Mitochondrial and nuclear DNA-repair capacity of various brain regions in mouse is altered in an age-dependent manner. *Neurobiol Aging*. In press.

22. Navarro A. Mitochondrial enzyme activities as biochemical markers of aging. *Mol Aspects Med*. 2004;25:37-48.

23. MacMillan-Crow LA, Crow JP, Thompson JA. Peroxynitrite-mediated inactivation of manganese superoxide dismutase involves nitration and oxidation of critical tyrosine residues. *Biochemistry*. 1998;37:1613-1622.

24. Chen JJ, Yu BP. Alterations in mitochondrial membrane fluidity by lipid peroxidation products. *Free Radic Biol Med*. 1994;17: 411-418.

25. Laganiere S, Yu BP. Modulation of membrane phospholipid fatty acid composition by age and food restriction. *Gerontology*. 1993;39:7-18.

26. Andreyev AY, Kushnareva YE, Starkov AA. Mitochondrial metabolism of reactive oxygen species. *Biochemistry (Mosc)*. 2005;70:200-214.

27. Petrosillo G, Ruggiero FM, Paradies G. Role of reactive oxygen species and cardiolipin in the release of cytochrome c from mitochondria. *FASEB J*. 2003;17:2202-2208.

28. Shidoji Y, Hayashi K, Komura S, Ohishi N, Yagi K. Loss of molecular interaction between cytochrome c and cardiolipin due to lipid peroxidation. *Biochem Biophys Res Commun*. 1999;264:343-347.

29. Crompton M. The mitochondrial permeability transition pore and its role in cell death. *Biochem J*. 1999;341:233-249.

30. Kroemer G, Dallaporta B, Resche-Rigon M. The mitochondrial death/life regulator in apoptosis and necrosis. *Annu Rev Physiol*. 1998;60:619-642.

31. Vieira HL, Belzacq AS, Haouzi D, et al. The adenine nucleotide translocator: a target of nitric oxide, peroxynitrite, and 4-hydroxynonenal. *Oncogene*. 2001;20:4305-4316.

32. Ott M, Robertson JD, Gogvadze V, Zhivotovsky B, Orrenius S. Cytochrome c release from mitochondria proceeds by a two-step process. *Proc Natl Acad Sci USA*. 2002;99:1259-1263.

33. Marzo I, Brenner C, Zamzami N, et al. Bax and adenine nucleotide translocator cooperate in the mitochondrial control of apoptosis. *Science*. 1998;281:2027-2031.

34. Green DR, Reed JC. Mitochondria and apoptosis. *Science*. 1998;281:1309-1312.

35. Li P, Nijhawan D, Budihardjo I, et al. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell*. 1997;91:479-489.

36. Erdelyi K, Bakondi E, Gergely P, Szabo C, Virag L. Pathophysiologic role of oxidative stress-induced poly(ADP-ribose) polymerase-1 activation: focus on cell death and transcriptional regulation. *Cell Mol Life Sci*. 2005;62:751-759.

37. Beal MF. Mitochondria, oxidative damage, and inflammation in Parkinson's disease. *Ann N Y Acad Sci*. 2003;991:120-131.

38. Levites Y, Weinreb O, Maor G, Youdim MB, Mandel S. Green tea polyphenol (-)-epigallocatechin-3-gallate prevents N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced dopaminergic neurodegeneration. *J Neurochem*. 2001;78:1073-1082.

39. Miller ER, III, Pastor-Barriuso R, Dalal D, Riemersma RA, Appel LJ, Guallar E. Meta-analysis: high-dosage vitamin E supplementation may increase all-cause mortality. *Ann Intern Med*. 2005;142:37-46.

40. Beal MF, Matthews RT. Coenzyme Q10 in the central nervous system and its potential usefulness in the treatment of neurodegenerative diseases. *Mol Aspects Med*. 1997;18 Suppl:S169-S179.

41. Day BJ. Catalytic antioxidants: a radical approach to new therapeutics. *Drug Discov Today*. 2004;9:557-566.

42. Kaul S, Kanthasamy A, Kitazawa M, Anantharam V, Kanthasamy AG. Caspase-3 dependent proteolytic activation of protein kinase C delta mediates and regulates 1-methyl-4-phenylpyridinium (MPP+)-induced apoptotic cell death in dopaminergic cells: relevance to oxidative stress in dopaminergic degeneration. *Eur J Neurosci*. 2003;18:1387-1401.

43. Pong K, Doctrow SR, Baudry M. Prevention of 1-methyl-4-phenylpyridinium- and 6-hydroxydopamine-induced nitration of tyrosine hydroxylase and neurotoxicity by EUK-134, a superoxide dismutase and catalase mimetic, in cultured dopaminergic neurons. *Brain Res*. 2000;881:182-189.

44. Jung C, Rong Y, Doctrow S, Baudry M, Malfroy B, Xu Z. Synthetic superoxide dismutase/catalase mimetics reduce oxidative stress and prolong survival in a mouse amyotrophic lateral sclerosis model. *Neurosci Lett*. 2001;304:157-160.

45. Peng J, Stevenson FF, Doctrow SR, Andersen JK. Superoxide dismutase/catalase mimetics are neuroprotective against selective paraquat-mediated dopaminergic neuron death in the substantial nigra: implications for Parkinson disease. *J Biol Chem*. 2005;280:29194-29198.

46. Petri S, Kiaei M, Kipiani K, et al. Additive neuroprotective effects of a histone deacetylase inhibitor and a catalytic antioxidant in a transgenic mouse model of amyotrophic lateral sclerosis. *Neurobiol Dis*. 2006;22:40-49.

47. Melov S, Schneider JA, Day BJ, et al. A novel neurological phenotype in mice lacking mitochondrial manganese superoxide dismutase. *Nat Genet*. 1998;18:159-163.

48. Schriner SE, Linford NJ, Martin GM, et al. Extension of murine life span by overexpression of catalase targeted to mitochondria. *Science*. 2005;308:1909-1911.

49. Sheu SS, Nauduri D, Anders MW. Targeting antioxidants to mitochondria: a new therapeutic direction. *Biochim Biophys Acta*. 2006;1762:256-265.

50. Murphy MP, Smith RA. Drug delivery to mitochondria: the key to mitochondrial medicine. *Adv Drug Deliv Rev*. 2000;41:235-250.

51. Jauslin ML, Meier T, Smith RA, Murphy MP. Mitochondria-targeted antioxidants protect Friedreich Ataxia fibroblasts from endogenous oxidative stress more effectively than untargeted antioxidants. *FASEB J*. 2003;17:1972-1974.

52. Dhanasekaran A, Kotamraju S, Kalivendi SV, et al. Supplementation of endothelial cells with mitochondria-targeted antioxidants inhibit peroxide-induced mitochondrial iron uptake, oxidative damage, and apoptosis. *J Biol Chem*. 2004;279:37575-37587.

53. Smith RA, Porteous CM, Gane AM, Murphy MP. Delivery of bioactive molecules to mitochondria in vivo. *Proc Natl Acad Sci USA*. 2003;100:5407-5412.

54. Adlam VJ, Harrison JC, Porteous CM, et al. Targeting an antioxidant to mitochondria decreases cardiac ischemia-reperfusion injury. *FASEB J*. 2005;19:1088-1095.

55. Smith RA, Porteous CM, Coulter CV, Murphy MP. Selective targeting of an antioxidant to mitochondria. *Eur J Biochem*. 1999;263:709-716.

56. Kelso GF, Porteous CM, Coulter CV, et al. Selective targeting of a redox-active ubiquinone to mitochondria within cells: antioxidant and antiapoptotic properties. *J Biol Chem*. 2001;276:4588-4596.

57. James AM, Cocheme HM, Smith RA, Murphy MP. Interactions of mitochondria-targeted and untargeted ubiquinones with the mitochondrial respiratory chain and reactive oxygen species. Implications for the use of exogenous ubiquinones as therapies and experimental tools. *J Biol Chem*. 2005;280:21295-21312.

58. Zhao K, Zhao GM, Wu D, et al. Cell-permeable peptide antioxidants targeted to inner mitochondrial membrane inhibit mitochondrial swelling, oxidative cell death, and reperfusion injury. *J Biol Chem*. 2004;279:34682-34690.

59. Szeto HH. Cell-permeable, mitochondrial-targeted, peptide antioxidants. *AAPS J*. 2006;8:E277-E283.

60. Winterbourn CC, Parsons-Mair HN, Gebicki S, Gebicki JM, Davies MJ. Requirements for superoxide-dependent tyrosine hydroperoxide formation in peptides. *Biochem J*. 2004;381:241-248.

61. Zhao K, Luo G, Zhao GM, Schiller PW, Szeto HH. Transcellular transport of a highly polar 3+ net charge opioid tetrapeptide. *J Pharmacol Exp Ther*. 2003;304:425-432.

62. Zhao K, Luo G, Giannelli S, Szeto HH. Mitochondria-targeted peptide prevents mitochondrial depolarization and apoptosis induced by tert-butyl hydroperoxide in neuronal cell lines. *Biochem Pharmacol*. 2005;70:1796-1806.

63. Drin G, Cottin S, Blanc E, Rees AR, Temsamani J. Studies on the internalization mechanism of cationic cell-penetrating peptides. *J Biol Chem*. 2003;278:31192-31201.

64. Derossi D, Calvet S, Trembleau A, Brunissen A, Chassaing G, Prochiantz A. Cell internalization of the third helix of the Antennapedia homeodomain is receptor-independent. *J Biol Chem*. 1996;271:18188-18193.

65. Szeto HH, Schiller PW, Zhao K, Luo G. Fluorescent dyes alter intracellular targeting and function of cell-penetrating tetrapeptides. *FASEB J*. 2005;19:118-120.

66. Haidara K, Morel I, Abalea V, Gascon BM, Denizeau F. Mechanism of tert-butylhydroperoxide induced apoptosis in rat hepatocytes: involvement of mitochondria and endoplasmic reticulum. *Biochim Biophys Acta*. 2002;1542:173-185.

67. Piret JP, Arnould T, Fuks B, Chatelain P, Remacle J, Michiels C. Mitochondria permeability transition-dependent tert-butyl hydroperoxide-induced apoptosis in hepatoma HepG2 cells. *Biochem Pharmacol*. 2004;67:611-620.

68. Byrne AM, Lemasters JJ, Nieminen AL. Contribution of increased mitochondrial free Ca2+ to the mitochondrial permeability transition induced by tert-butylhydroperoxide in rat hepatocytes. *Hepatology*. 1999;29:1523-1531.

69. Nieminen AL, Byrne AM, Herman B, Lemasters JJ. Mitochondrial permeability transition in hepatocytes induced by t-BuOOH: NAD(P)H and reactive oxygen species. *Am J Physiol*. 1997;272:C1286-C1294.

70. Pias EK, Ekshyyan OY, Rhoads CA, Fuseler J, Harrison L, Aw TY. Differential effects of superoxide dismutase isoform expression on hydroperoxide-induced apoptosis in PC-12 cells. *J Biol Chem*. 2003;278:13294-13301.

71. Szeto HH, Lovelace JL, Fridland G, et al. In vivo pharmacokinetics of selective mu-opioid peptide agonists. *J Pharmacol Exp Ther*. 2001;298:57-61.

72. Zhao GM, Wu D, Soong Y, et al. Profound spinal tolerance after repeated exposure to a highly selective mu-opioid peptide agonist: role of delta-opioid receptors. *J Pharmacol Exp Ther*. 2002;302:188-196.

73. Przyklenk K. Pharmacologic treatment of the stunned myocardium: the concepts and the challenges. *Coron Artery Dis*. 2001;12:363-369.

74. Masini E, Cuzzocrea S, Mazzon E, Marzocca C, Mannaioni PF, Salvemini D. Protective effects of M40403, a selective superoxide dismutase mimetic, in myocardial ischaemia and reperfusion injury in vivo. *Br J Pharmacol*. 2002;136:905-917.

75. Mackensen GB, Patel M, Sheng H, et al. Neuroprotection from delayed postischemic administration of a metalloporphyrin catalytic antioxidant. *J Neurosci*. 2001;21:4582-4592.

76. Wu D, Soong Y, Zhao GM, Szeto HH. A highly potent peptide analgesic that protects against ischemia-reperfusion-induced myocardial stunning. *Am J Physiol Heart Circ Physiol*. 2002;283: H783-H791.

77. Song W, Shin J, Lee J, et al. A potent opiate agonist protects against myocardial stunning during myocardial ischemia and reperfusion in rats. *Coron Artery Dis*. 2005;16:407-410.

78. Cho J, Won K, Wu D, et al. Potent mitochondria-targeted peptides reduce myocardial infarction in rats. *Coron Artery Dis*. 2006; In press.

79. Cho S, Szeto HH, Kim HJ, Pinto J. *A cell permeable antioxidant peptide SS31 attenuates CD36-mediated ischemic injury via normalizing redox state [abstract]*. Washington, DC: Society for Neuroscience; 2005.

80. Vijayvergiya C, Beal MF, Buck J, Manfredi G. Mutant superoxide dismutase 1 forms aggregates in the brain mitochondrial matrix of amyotrophic lateral sclerosis mice. *J Neurosci*. 2005;25:2463-2470.

81. Petri S, Kiaei M, Damiano M, et al. Cell-permeable peptide antioxidants as a novel therapeutic approach in a mouse model of amyotrophic lateral sclerosis. *J Neurochem*. 2006;98: 1141-1148.