ANTIOXIDANTS & REDOX SIGNALING
Volume 10, Number 3, 2008

DOI: 10.1089/ars.2007.1892

# Forum Review

# Mitochondria-Targeted Cytoprotective Peptides for Ischemia–Reperfusion Injury

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## ABSTRACT

**It is now recognized that oxidative injury and mitochondrial dysfunction are responsible for many clinical disorders with unmet needs, including ischemia–reperfusion injury, neurodegeneration, and diabetes. Mitochondrial dysfunction can lead to cell death by apoptosis or necrosis. As mitochondria are the major source of intracellular reactive oxygen species (ROS), and mitochondria are also the primary target for ROS, the ideal drug therapy needs to be targeted to mitochondria. A number of approaches have been used for targeted delivery of therapeutic agents to mitochondria. This review will focus on a novel class of cell-permeable small peptides (Szeto–Schiller peptides) that selectively partition to the inner mitochondrial membrane and possess intrinsic mitoprotective properties. Studies with isolated mitochondrial preparations and cell cultures show that these SS peptides can scavenge ROS, reduce mitochondrial ROS production, and inhibit mitochondrial permeability transition. They are very potent in preventing apoptosis and necrosis induced by oxidative stress or inhibition of the mitochondrial electron transport chain. These peptides have demonstrated excellent efficacy in animal models of ischemia–reperfusion, neurodegeneration, and renal fibrosis, and they are remarkably free of toxicity. The pharmacology of the SS peptides in models of ischemia—reperfusion will be the focus of this review.** *Antioxid. Redox Signal.* 10, 601–619.

## INTRODUCTION

MITOCHONDRIA HAVE RECENTLY entered center stage as the major regulator of life and death in the cell. In addition to their major role as energy providers for cellular processes, mitochondria are also the major producers of intracellular reactive oxygen species (ROS) (6, 103). Cells die from necrosis when they cannot maintain adequate ATP levels (43, 48). On the other hand, oxidative damage to mitochondria can result in cytochrome c release into the cytosol and activation of the caspase cascade, leading to apoptosis (72, 73, 77). Intracellular ATP serves as a switch between apoptosis and necrosis because apoptosis involves energy-requiring steps (48, 56). Cell death induced by mitochondrial oxidative damage plays an important role in numerous clinical disorders, including ischemia—reperfusion injury, neurodegenerative disorders, diabetes, inflammatory disorders, drug-induced toxicity, and age-related degenerative diseases (7, 11, 37, 44, 110). Many clinically approved drugs, including doxorubicin and paclitaxel, act directly on mitochondria to induce apoptosis (86). Minimizing mitochondrial oxidative stress and protecting mitochondrial function can potentially provide therapeutic benefits to this vast group of diseases with unmet needs. There are several hurdles in the quest for mitoprotective drugs. The difficulties include delivery of drugs to mitochondria, minimization of adverse effects, and delivering drugs across the blood-brain barrier. This review will summarize some of the approaches that have been used for targeted delivery of conventional therapeutic agents to the mitochondrial matrix. The major focus of this review, however, will be on a novel class of cell-permeable small peptides (Szeto—Schiller peptides) that selectively partition to the inner mitochondrial membrane and possess intrinsic mitoprotective properties (98, 99). This review will summarize results from cell culture studies that show the extraordinary potency of these

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SS peptides in preventing apoptosis and necrosis induced by oxidative stress or inhibition of mitochondrial respiration. A summary of results from *ex vivo* and *in vivo* animal models will also be presented, with special emphasis on ischemia–reperfusion injury. The efficacy of these peptides as neuroprotective agents was recently reviewed (99).

## MITOCHONDRIA AS GATEKEEPER OF CELL SURVIVAL

Long considered the powerhouses of our cells, mitochondria are responsible for the enormous task of supplying intracellular ATP via oxidative phosphorylation. Mitochondria produce more than 90% of our cellular energy under normal conditions. Energy production results from the tricarboxylic acid (TCA) cycle and the electron transport chain (ETC). The TCA cycle provides reducing equivalents in the form of NADH and FADH that then enter into the ETC. As the electrons donated from NADH and FADH flow from Complex I through Complex IV of the ETC, protons are pumped into the intermembrane space to establish a protonomotive force, which is used by Complex V to phosphorylate ADP to ATP by the $F_0F_1$ ATP synthase.

Oxygen normally serves as the ultimate electron acceptor in the ETC and is reduced to water. However, electron leak to oxygen through complex I and complex III can generate superoxide anion ($O_2^{\cdot -}$)[see reviews, (6, 103)]. The rate of $O_2^{\cdot -}$ 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 (2). The location of $O_2^{\cdot -}$ within mitochondria is important because $O_2^{\cdot -}$ does not diffuse readily across membranes. Recent studies suggest that complex I produces $O_2^{\cdot -}$ into the matrix, while complex III can release $O_2^{\cdot -}$ into the matrix as well as the intermembrane space (62). The disposition of mitochondrial ROS was reviewed recently (6). Superoxide anion can be eliminated by dismutation carried out by the mitochondrial matrix enzyme Mn superoxide dismutase (MnSOD), or by the CuZnSOD in the intermembrane space, to $H_2O_2$. Although $H_2O_2$ may freely diffuse out of mitochondria, it can be readily inactivated by catalase or glutathione reductase. The inner membrane also contains many redox-active transition metals such as iron and copper that can convert $H_2O_2$ to the highly reactive hydroxyl radical (OH·) via the Fenton reaction. OH· may further react with bicarbonate to yield the very reactive carbonate radical anion. $O_2^{\cdot -}$ can also react with another free radical nitric oxide formed by mitochondrial nitric oxide synthase to generate the highly reactive peroxynitrite ($ONOO^-$).

Mitochondria are normally protected from oxidative damage by a multilayer network of mitochondrial antioxidant systems, including glutathione peroxidase, thioredoxin, glutaredoxin, and catalase [see review (6)], but they can undergo oxidative damage when ROS production exceeds the antioxidant capacity of mitochondria. ROS can initiate damage to nucleic acids, proteins, and lipids in mitochondria. Mitochondrial DNA is particularly susceptible to oxidative damage because they lack histones and have much limited base excision repair mechanisms compared to nuclear repair mechanisms (82). Protein oxidation and nitration result in altered function of many enzymes in the mitochondrial ETC, including NADH dehydrogenase, NADH oxidase, cytochrome c oxidase, and ATPase (12, 66), while oxidation of the adenine nucleotide translocator impairs the influx of ADP into the matrix for ATP synthesis (59).

Besides reduced function of mitochondrial enzymes, mitochondrial ROS can also cause lipid peroxidation that can lead to mitochondrial dysfunction. Cytochrome c is normally bound to the inner mitochondrial membrane by association with cardiolipin. Peroxidation of cardiolipin leads to loss of cardiolipin on the inner membrane and dissociation of cytochrome c (77, 93). This will lead to compromised function of cytochrome c oxidase, reduced ATP production, and increased ROS generation (14). Cytochrome c, once dissociated from the inner membrane, can then be released through the outer mitochondrial membrane into the cytosol (93).The mechanisms by which cytochrome c may be released through the outer membrane, termed mitochondrial outer membrane permeability (MOMP), was reviewed recently (27). One mechanism is believed to 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 (VDAC) on the outer membrane and the adenine nucleotide translocator (ANT) on the inner membrane (19). Opening of the MPT pore causes a sudden increase in permeability of the inner mitochondrial membrane. This will result in swelling of the mitochondrial matrix, rupture of the outer membrane, and release of cytochrome c (44, 74). Mitochondrial permeability transition can be induced by calcium ($Ca^{2+}$) overload and high concentrations of inorganic phosphate (Pi), conditions commonly associated with ischemia (see later section on ischemia–reperfusion). ROS can further promote MPT by causing oxidation of thiol groups on the ANT (104). Cytochrome c release may also occur via MPT-independent mechanisms, and may involve an oligomeric form of Bax (42, 87). Certain death signals activate the pro-apoptotic protein Bax in the cytoplasm, resulting in translocation to mitochondria, and oligomerization of Bax on the outer membrane leads to the release of cytochrome c into the cytoplasm.

Cytochrome c in the cytosol binds to apoptotic protease activating factor-1 (Apaf-1) to initiate the formation of an apoptosome, which then binds pro-caspase-9 (54). The oligomerization of caspase-9 on the apoptosome activates the protease. The active caspase-9 cleaves two "executioner" caspases, caspase-3 and caspase-7, that then go on to cleave key substrates within the cell (29, 54, 121). This has been termed the intrinsic mitochondrial pathway of apoptosis. Mitochondrial dysfunction may therefore lead to necrosis or apoptosis (Fig. 1). Necrosis is characterized by cell swelling and disruption of the cell membrane, leading to release of cellular contents, especially proteolytic enzymes, which may result in destruction of neighboring cells. Apoptosis, on the other hand, is defined as programmed cell death wherein the organism eliminates senescent, abnormal cells without affecting surrounding cells, and is deemed to be preferable for the survival of the organism since it eliminates dying cells by phagocytosis. The decision step between death by apoptosis or necrosis appears to be dependent on intracellular ATP content (48). Apoptosis involves energy-requiring steps, especially in the formation of the apoptosome complex between Apaf-1 and cytochrome c (56, 121). Thus in

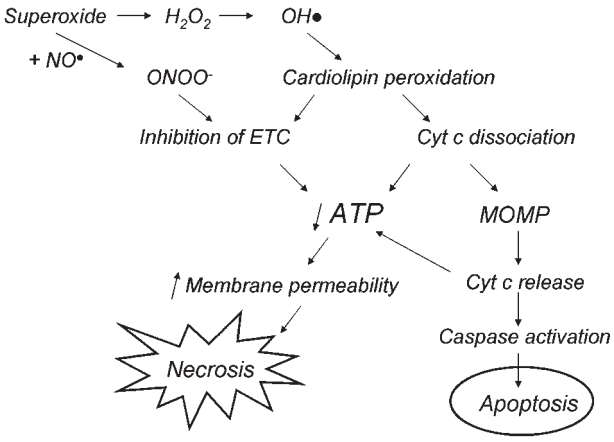


**FIG. 1. A scheme showing mitochondrial ROS production resulting in cell death by necrosis or apoptosis.** Protein oxidation and nitration impairs the function of many enzymes in the mitochondrial electron transport chain (ETC) and decreases ATP production. Peroxidation of cardiolipin leads to loss of cardiolipin on the inner membrane and dissociation of cytochrome c. This will lead to compromised function of cytochrome c oxidase, further reduction in ATP production, and increased ROS generation. Cytochrome c, once dissociated from the inner membrane, can be released through the outer mitochondrial membrane into the cytosol. Mitochondrial outer membrane permeability (MOMP) can be elicited by mitochondrial permeability transition (MPT) or may involve an oligomeric form of Bax forming a pore on the outer membrane. Cytochrome c in the cytosol binds to Apaf-1 to initiate the formation of an apoptosome, followed by activation of the caspase cascade to cause cell death by apoptosis. In the event of significant cellular ATP depletion, death can only occur by necrosis because ATP is required for the apoptosis pathway.

the event of significant cellular ATP depletion, death can only occur by necrosis. The inhibition of ATP-dependent ion pumps in the plasma membrane can lead to the opening of a "death channel" that is selectively permeable to anions, resulting in cytoplasmic membrane swelling and rupture (69). Thus caspases not only mediate apoptosis but also protect against necrosis.

## THERAPEUTIC TARGETS FOR MINIMIZING CELL DEATH INDUCED BY MITOCHONDRIAL OXIDATIVE DAMAGE

As proposed in Fig. 1, a number of cellular targets are available for therapeutic development to prevent cell death caused by mitochondrial oxidative damage. Caspase inhibitors can reduce apoptosis, but would merely switch the morphology of cell death to necrosis, which in the whole organism would be a worse outcome (80, 85). On the other hand, PARP [poly-(ADP-ribose)-polymerase] inhibitors will help to preserve ATP and switch the mode of cell death from necrosis to apoptosis (85). To reduce total cell death, it is necessary to target upstream of cytochrome c release. This may be achieved with MPT inhibitors that directly inhibit the formation of the pore. MPT inhibitors such as cyclosporin A and trifluoperazine have been shown to reduce MPT, decrease intracellular ROS, and reduce cell death in cultured cells exposed to oxidative stress such as *tert*-butylhydroperoxide or inhibitors of complex I of the electron transport chain (46, 49). Unfortunately, side effects associated with these MPT inhibitors limit their therapeutic potential. It is possible to target even further upstream by reducing mitochondrial oxidative stress since ROS play an important role in MPT pore opening. The endogenous antioxidant proteins such as SOD or catalase do not penetrate cell membranes and are therefore ineffective against intracellular ROS. Vitamin E or coenzyme Q (CoQ) are very lipophilic and tend to be retained in cell membranes and fail to achieve significant intracellular concentrations. *N*-acetylcysteine (NAC), a thiol-antioxidant, can replenish the endogenous antioxidant glutathione and is very effective in reducing oxidative cell death but must be given in m*M* concentrations in cultured cells. *In vivo*, however, NAC failed to provide significant antioxidant effect, presumably due to its low lipid solubility and tissue distribution (17). To overcome the limitations of natural antioxidants, a number of low molecular weight catalytic antioxidants, generally referred to as SOD mimetics, have been developed [see review (21)]. Several of these SOD mimetics have been reported to be effective in blocking oxidant stress in cell models, including protection against ionization radiation (47) and inhibition of staurosporine-induced apoptosis (79). Studies have shown that these SOD mimetics can provide some protection against ischemia—reperfusion injury (28) and neurodegenerative diseases such as amyotrophic lateral sclerosis (39), and several of these are now in clinical development [see review, (64)]. However, while these SOD mimetics are cell-permeable, they do not selectively target mitochondria and they only act on superoxide. It should be mentioned that some of the spin trap agents such as TEMPOL (2,2,6,6-tetramethylpiperidine-1-oxyl) can have prooxidant activity and can be cytotoxic ($IC_{50} \sim 0.1$ to 100 m*M*) (36, 70). Interestingly, it appears that TEMPOL distributes to mitochondria and can inhibit complex I of the ETC, resulting in reduced ATP production and increased ROS generation (61).

## TARGETED DELIVERY OF THERAPEUTICS TO MITOCHONDRIA

Improved efficacy and reduced side effects can be achieved with molecules that selectively target and concentrate in mitochondria. This is particularly important since superoxide is formed by complex I and complex III on the mitochondria inner membrane, and peroxidation of cardiolipin on the inner membrane is the leading step towards reduced ATP production, MPT, and cytochrome c release (see Fig. 1). Thus in order to prevent cell death caused by ROS-induced mitochondrial damage, it is important to deliver the treatment agent to mitochondria. This idea is reinforced by the recent report that overexpression of catalase in mitochondria increased lifespan by 20% in mice, whereas overexpression of catalase in peroxisomes had no significant effect (90). Specific targeting of the treatment molecule to mitochondria can also minimize adverse effects. For example, cyclosporin A has very high affinity for cyclophilin D and is a potent inhibitor of MPT. However, cy-

closporin A targets at least eight other cyclophilins in the cell which can result in unwanted side effects (105). These side effects would be minimized if cyclosporine could be targeted to mitochondria, so that high drug levels can be attained at the site of action while minimizing drug exposure elsewhere.

A number of approaches have been used to target molecules to mitochondria. They can be divided into two general approaches: (i) mitochondrial potential-dependent methods; and (ii) mitochondrial potential-independent methods.

## *Mitochondrial potential-dependent methods*

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 150–180 mV is generated across the inner membrane. Depending on their charge, lipophilic cations may accumulate 100–1000-fold in the mitochondrial matrix.

*TPP+-conjugated antioxidants.* Murphy and coworkers applied this approach by conjugating the triphenylalkylphosphonium cation ($TPP^+$) to lipophilic antioxidants such as CoQ (MitoQ). MitoQ is taken up into the mitochondrial matrix in a potential-dependent manner, and is effluxed out of mitochondria upon depolarization. Within the mitochondria, MitoQ is thought to be accumulated on the matrix side of the inner membrane. The theoretical background on the development of these molecules and much of the work done to date have been extensively summarized in a recent review [see review, (63)]. A series of $TPP^+$-conjugates have been designed to reduce superoxide (MitoSOD), hydrogen peroxide (MitoPeroxidase), ferrous ion (MitoTEMPOL), and lipid peroxidation (MitoQ and MitoE).

The accumulation of these lipophilic cations in the mitochondrial matrix can disrupt mitochondrial potential and inhibit mitochondrial respiration and ATP production. As a result, the therapeutic index of these molecules is rather low, with toxic concentrations being only ~10-fold greater than effective concentrations. This may be even more of a problem with MitoTEMPOL considering the intrinsic mitotoxic effects of TEMPOL (36, 70). The utility of $TPP^+$-conjugated antioxidants may also be limited by their requirement of mitochondrial potential for mitochondrial uptake. Diseased mitochondria are unlikely to have normal mitochondrial potential. Furthermore, the uptake of these antioxidants are self-limiting in that uptake is reduced at concentrations $>50\ \mu M$ when the accumulated cations in the matrix begin to depolarize mitochondria (96).

By preferentially accumulating in the mitochondrial matrix, these TPP+-conjugated antioxidants are more potent than their lipophilic counterparts in reducing intracellular ROS, preserve reduced thiols, and reduce apoptosis in cultured cells [see review, (63)]. This enhanced potency of the $TPP^+$-conjugated antioxidants was abolished in cells pretreated with the uncoupler FCCP (carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone), confirming their dependence on mitochondrial potential for uptake. The conjugation of a nitroxide to $TPP^+$ (MitoCP) effectively inhibited $H_2O_2$ and lipid peroxide-induced oxidative stress and apoptosis while the unconjugated nitroxide was ineffective (23). However, $TPP^+$-conjugated TEMPOL and EUK-134 failed to inhibit staurosporin-induced apoptosis, and did not improve on the ability of the unconjugated compounds to inhibit selenium-induced apoptosis (22).

In contrast to the extensive work that has been done on the biochemistry of these compounds, there is relatively little data to support their *in vivo* efficacy in animal models of clinical disorders. Pretreatment of rats with MitoQ in their drinking water for 2 weeks led to protection of myocardial function in an *ex vivo* myocardial ischemia—reperfusion model (3). However, there is no evidence that MitoQ will be effective in an *in vivo* model, especially without prolonged pretreatment prior to ischemia. A recent article reported that MitoE did not protect striatal medium spiny neurons in a model of perinatal ischemia—reperfusion injury (18). Based on this finding, the authors concluded that oxidative stress does not play an important role in this model of ischemia—reperfusion. A more likely explanation is inadequate diffusion of MitoE across the blood—brain barrier, or limited uptake of this lipophilic cation by compromised mitochondria. A study with Drosophila also showed that MitoQ had no effect on the normal aging process (58). More *in vivo* animal studies are needed to demonstrate the advantages of this approach for mitochondrial targeting.

*Choline esters of glutathione and N-acetyl-cysteine.* Glutathione (L-γ-glutamyl-L-cysteinylglycine) 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, Sheu and coworkers prepared choline esters of glutathione and *N*-acetyl-cysteine to enhance their uptake into mitochondria (92). Early studies show that they can protect against oxidative stress in cultured cells, but *in vivo* animal studies are not yet available.

*Liposome and liposome-like vesicles.* Exploring amphiphilic cations with a delocalized positive charge center known to accumulate inside mitochondria, Weissig and coworkers selected dequalinium to prepare a colloidal delivery system specific for mitochondria [see review, (109)]. Dequalinium is a dicationic compound, a symmetrical molecule with two cationic charge centers separated by a hydrophobic carbon chain. This molecule can self-assemble in aqueous medium into stable vesicles, which have been termed DQAsomes. These DQAsomes penetrate cells via endocytosis, and must subsequently be released from the endosomes before they can deliver the "cargo" to mitochondria. Fortunately, cationic lipids are known to exert a destabilizing effect on endosomal membranes, and Weissig and co-workers have been able to deliver DNA into mitochondria using these DQAsomes (20). However, most of the liposomes made from dequalinium analogs are cytotoxic. This approach to deliver drugs or DNA into mitochondria is still in early stages of development, and further structure—activity studies will be required to optimize a delivery system based on dequalinium.

## *Mitochondrial potential-independent methods*

*Nitroxide radicals conjugated to gramicidin S peptides.* Although nitroxides like TEMPOL have antioxi-

dant and electron scavenging properties, the required high concentrations (m*M*) limit *in vivo* efficacy. There have been attempts to improve the targeting of nitroxides to mitochondria using peptide vectors such as gramicidin S (GS). Gramicidin peptides are 15 residue peptides isolated from *Bacillus brevis*, and GS is unique in that it has a cyclic peptide chain. Gramicidin peptides assemble as a $\beta$-helix inside lipid bilayers. Although the 15 residue peptides are not long enough to span the membrane layer, they can dimerize to form an elongated channel that spans the membrane (5). Wipf and co-workers conjugated a pentapeptide sequence (Leu-D-Phe-Pro-Val-Orn) from GS to 4-amino-TEMPO (4-AT) to enhance mitochondrial targeting. When tested at a concentration of 10 $\mu M$, the GS peptide-conjugated 4-AT significantly reduced intracellular superoxide generation, caspase activation, and DNA fragmentation elicited by actinomycin D (112). In contrast, 4-AT itself had no effect. A recent article showed that a modified peptide analog (D-Phe-Pro-Val-Orn-Leu) is also effective in enhancing the delivery of 4-AT (38). At the effective concentration of 10 $\mu M$, these GS-conjugated nitroxides had no effect on intracellular ATP.

In addition to actinomycin D, uroepithelial cells were protected against radiation treatment if pretreated with 100 $\mu M$ of GS-conjugated 4-AT, whereas the unconjugated 4-AT was not effective (40). In the first *in vivo* study, treatment with a GS-conjugated TEMPOL derivative (XJB-5-131) (2 $\mu$mol/kg) prolonged survival of rats subjected to hemorrhagic shock (57). This approach of using gramicidin peptides provides an alternative to the lipophilic cation approach for targeted mitochondrial delivery.

*Mitochondria-targeted small peptides.* In contrast to the above approaches which all use a targeted delivery system to deliver conventional antioxidants to mitochondria, there is a novel class of cell-permeable small peptides that selectively partition into the inner mitochondrial membrane and possess intrinsic mitoprotective activities. The rest of this review will focus on the design, development, characterization, and evaluation of these unique molecules as mitoprotective and cytoprotective agents.

## MITOCHONDRIA-TARGETED CYTOPROTECTIVE PEPTIDES

### *Chemistry and cell uptake of SS peptides*

These novel peptides were originally designed by Hazel H. Szeto and Peter W. Schiller, hence they have been designated SS peptides. They are small, water-soluble, peptides limited to $<$10 amino acid residues. The chemical structures of some of the SS peptides are shown in Fig. 2. The synthesis of these peptides using solid phase methods has been described in previous publications (88, 89).

Common to all of these peptides is an alternating aromatic-cationic motif, with the basic amino acid residues (such as Arg and Lys) providing two positive charges. The free amine of the N-terminus of these peptides provides a third positive charge because the C-terminus has been amidated. This aromatic-cationic motif allows them to freely penetrate cells despite carrying a 3+ net charge at physiologic pH (119). When intestinal epithelial cells (Caco-2) were incubated with [$^3$H]SS-02, the radiolabel was detected in cell lysate as early as 5 min, and steady state levels were achieved by 30 min, suggesting that the peptide was diffusing freely in and out of the cell. The rate of uptake of [$^3$H]SS-02 was found to be concentration dependent over a wide range of concentrations with no evidence of saturability. Surprisingly, the uptake of SS-02 was not temperature dependent, and did not involve PEPT1, the peptide transporter that is highly expressed in Caco-2 cells (119). Similar uptake of SS-02 was observed with a variety of cell types, including neuronal cells (SH-SY5Y and $N_2A$ cells), renal epithelial cells (MDCK), endothelial cells (HUVEC), and human embryonic kidney cell line (HEK 293) (119). The specific amino acid sequence does not appear to be important, as SS-31 was also readily taken up by neuronal $N_2A$ cells (118). Cellular uptake of SS-02 was confirmed with the use of a fluorescent-labeled peptide and the microscopic image clearly showed intracellular distribution (119, 120). Furthermore, SS-02 was able to translocate across a monolayer of Caco-2 cells from the apical side to the basolateral side (119). The apparent permeability coefficient of SS-02 was calculated to be $1.24 \times 10^{-5}$

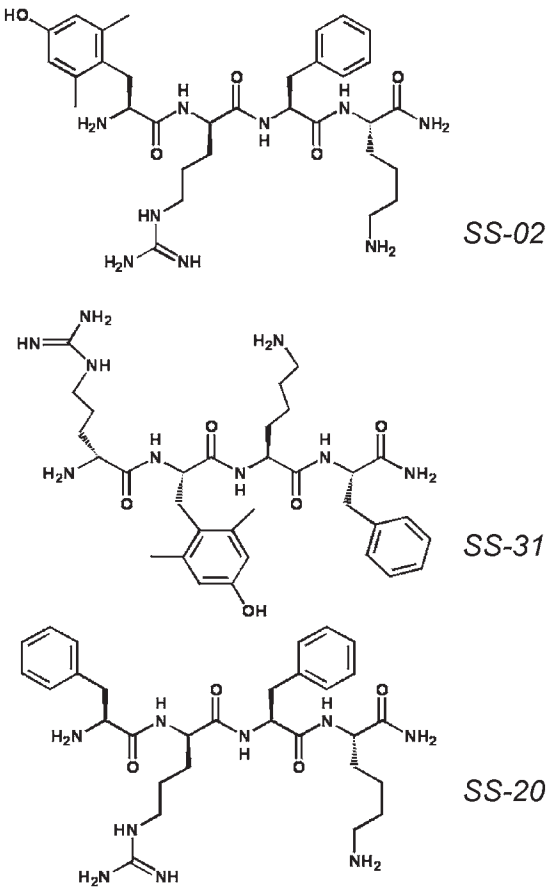


**FIG. 2. Chemical structures of SS peptides.** SS-02 (Dmt-D-Arg-Phe-Lys-$NH_2$)l; SS-31 (D-Arg-Lys-Phe-$NH_2$); SS20 (Phe-D-Arg-Phe-Lys-$NH_2$). Dmt = 2′,6′-dimethyltyrosine.

cm/s, which is comparable to that for the dipeptide Gly-Sar ($1.26 \times 10^{-5}$ cm/s) that is known to be taken up by PEPT1. In contrast, this permeability coefficient is ~1000-fold greater than for met-enkephalin, a pentapeptide that does not have any positive charges. On the other hand, an arginine tetramer penetrates poorly, and the larger cationic peptides (such as Tat and penetratin) enter by endosomal uptake (24, 81). The relative ease with which SS-02 penetrates cell membranes is unprecedented for a 3+ net charge peptide, and it appears that the aromatic-cationic motif provides a unique and highly favorable structure for membrane penetration.

### *Mitochondrial uptake of SS peptides*

The first evidence of mitochondrial uptake of these SS peptides came from confocal microscopic studies with a fluorescent-labeled SS-02 analog (SS-19) (120). Intestinal epithelial cells (Caco-2) were incubated with SS-19, and fluorescence was detected in the cytoplasm of all cells within 15 min, but the peptide was entirely excluded from the nucleus. The fluorescent label showed a perinuclear distribution that resembled the intracellular distribution of Mitrotracker TMRM, suggesting that the peptide was localized to mitochondria. Mitochondrial uptake of SS-02 and SS-31 were confirmed using isolated mouse liver mitochondria, and uptake of [$^3$H]SS-02 and [$^3$H]SS-31 were rapid with maximal levels reached within 2 min (118, 120). The fraction of peptide partitioned to mitochondria was estimated to be 1000–5000-fold compared to extramitochondrial concentration. Even though these are cationic peptides, mitochondrial fractionation studies revealed that the peptides are localized to the inner mitochondrial membrane rather than in the matrix (Fig. 3) (120). Contrary to MitoQ and MitoE, the uptake of these aromatic-cationic peptides into mitochondria is not dependent on mitochondrial potential, as the extent of uptake was only reduced by ~10–15% in mitochondria that were depolarized with FCCP (120). Because these positive-charged peptides are not delivered into the mitochondrial matrix, their uptake is not limited to mitochondria with normal potential. This is a major advantage when dealing with diseased mitochondria with compromised mitochondrial potential.

### *Tyrosine-containing SS peptides can scavenge ROS and inhibit lipid peroxidation*

The structure of SS-02 (Dmt-D-Arg-Phe-Lys-$NH_2$; Dmt = 2′,6′-dimethyltyrosine) and SS-31 (D-Arg-Dmt-Lys-Phe-$NH_2$) provide free radical scavenging abilities to these peptides. Tyrosine-containing analogs can dose-dependently scavenge

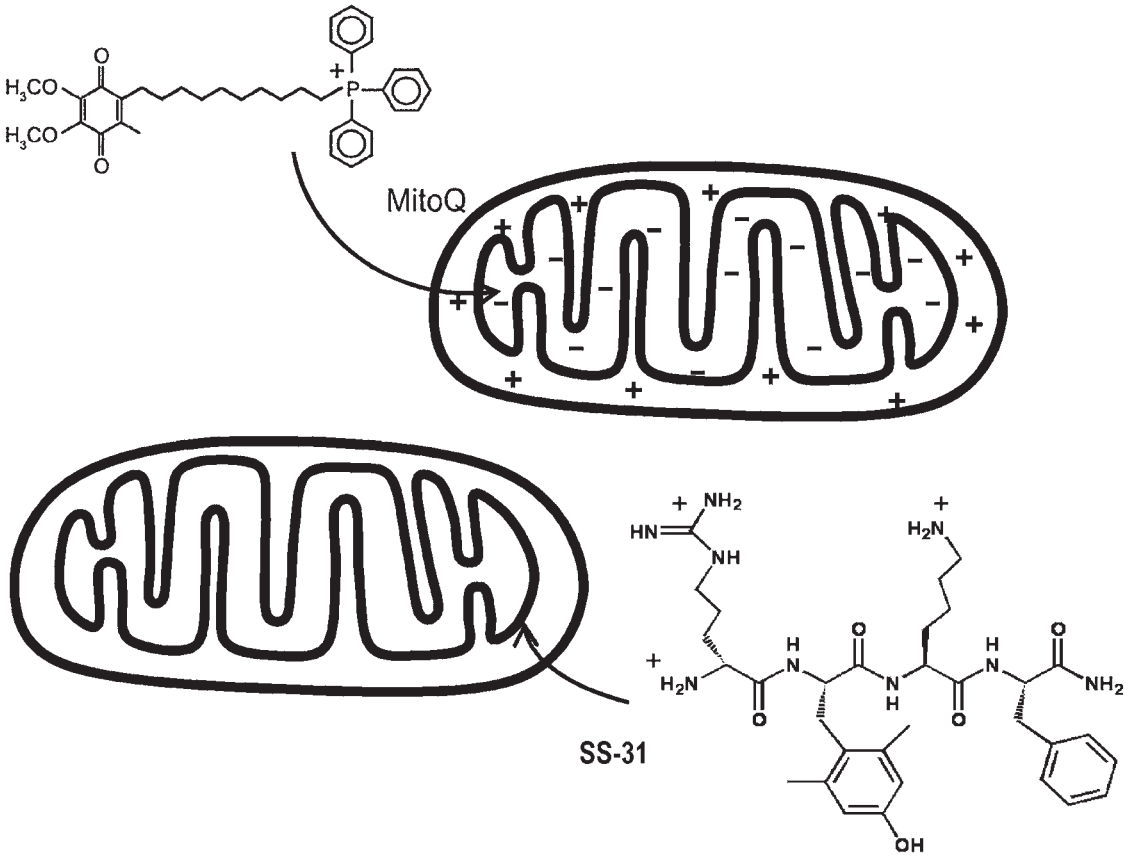


**FIG. 3. Targeted delivery of compounds to mitochondria.** MitoQ [Coenzyme Q conjugated to TPP+ (triphenylphosphonium cation)] is targeted into the mitochondrial matrix in a potential-driven manner. SS-31 and other SS peptide analogs target the inner mitochondrial membrane and concentrate more than 1000-fold. The mitochondrial uptake of SS peptides is not dependent on mitochondrial potential.

$H_2O_2$, $OH^{\cdot}$, and $ONOO^-$ (Fig. 4). The specific location of the tyrosine or dimethyltyrosine (Dmt) residue did not seem to be important as SS-31 was found to be as effective as SS-02 in scavenging all three ROS. However, replacement of tyrosine with phenylalanine (SS-20; Phe-D-Arg-Phe-Lys-$NH_2$) eliminated all scavenging ability, suggesting that the phenolic group on tyrosine mediates the scavenging activity (120). Tyrosine is known to scavenge oxyradicals, forming relatively unreactive tyrosyl radicals, which can be followed by radical—radical coupling to give dityrosine, or react with superoxide to form tyrosine hydroperoxide (111). Dimethyltyrosine was found to be more effective than tyrosine in scavenging $H_2O_2$, and Dmt-containing peptides were more effective than the tyrosine counterparts. By scavenging $OH^{\cdot}$, SS-31 and SS-02, but not SS-20, also inhibited lipid peroxidation, as demonstrated by the reduction in conjugated dienes formed from linoleic acid (Fig. 4) (120).

### *SS peptides reduce mitochondrial ROS production*

By targeting the inner mitochondrial membrane, the SS peptides are ideally located to reduce mitochondrial oxidative stress. Indeed, spontaneous $H_2O_2$ production by isolated guinea pig cardiac mitochondria was dose-dependently reduced by SS-31 and SS-02 (Fig. 5). Similar findings were obtained with mouse liver mitochondria (120). 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 (99). Furthermore, SS-31 and SS-02 can reduce mitochondrial $H_2O_2$ production in response to antimycin A (120). Antimycin A binds to complex III and inhibits the oxidation of ubiquinol, thereby generating large amounts of superoxide, which can then undergo dismutation by SOD to $H_2O_2$. Although SS-20 also produced a statistically significant de-

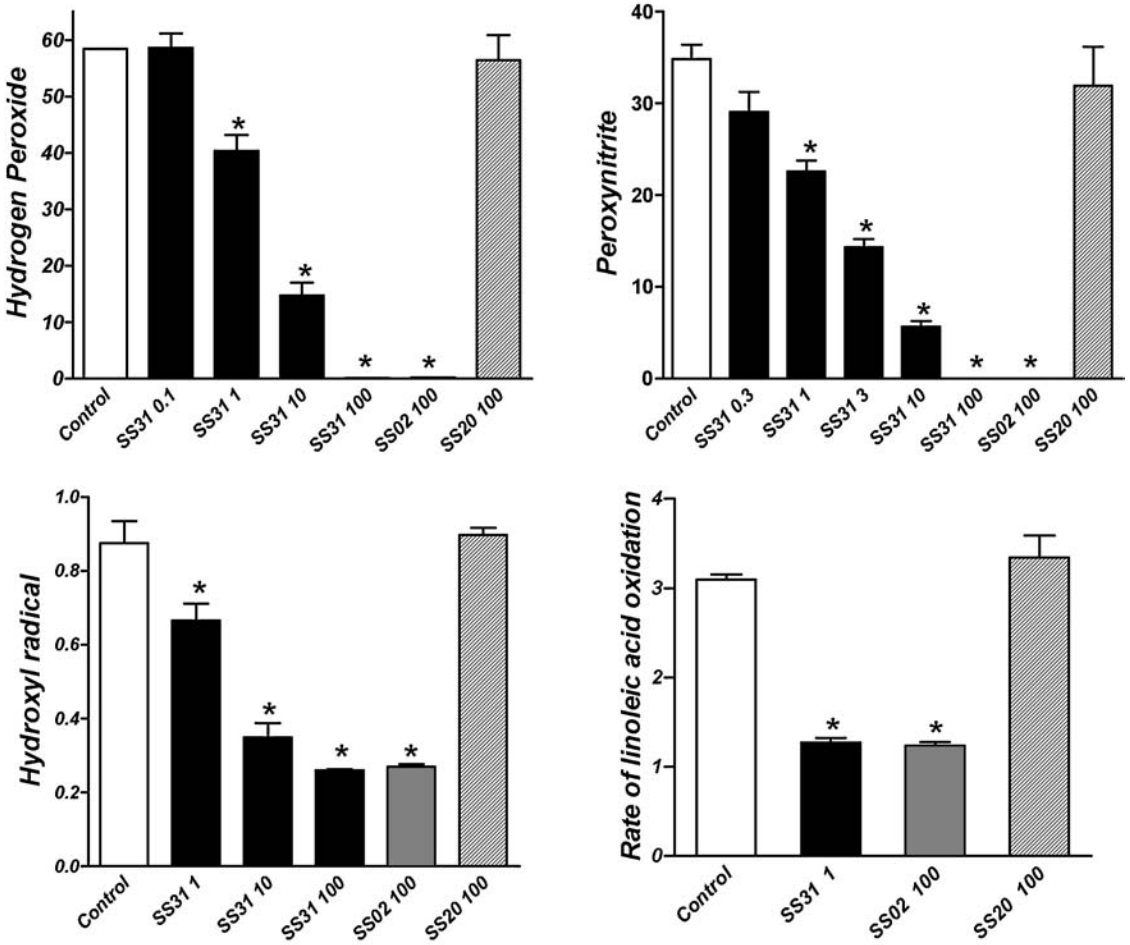


**FIG. 4. Antioxidant properties of SS peptides.** SS-31 (*black bar*) and SS-02 (*gray bar*) dose-dependently scavenge hydrogen peroxide ($H_2O_2$), peroxynitrite ($ONOO^-$), and hydroxyl radical ($OH^{\cdot}$). Hydrogen peroxide was measured by luminol chemiluminescence in the presence of horseradish peroxidase (HRP). Peroxynitrite was generated by thermal decomposition of SIN-1 and measured by luminal chemiluminescence. The hydroxyl radical was generated by the Fento reaction in the presence of deoxyribose, $H_2O_2$, and $FeCl_2$. Linoleic acid peroxidation was induced by 2,2′-azobis(2-amidinopropane) and detected by the formation of conjugated dienes measured by absorbance at 234 nm. SS-20 (*cross-hatched bar*) does not scavenge ROS and does not inhibit lipid peroxidation. $^*p < 0.05$ compared to control.

crease in $H_2O_2$ generation, the magnitude of effect was much less compared to SS-31 or SS-02 (Fig. 5). Since SS-20 does not scavenge $H_2O_2$ (Fig.4), these results suggest that SS20, and probably the other SS peptides, may have an independent action on reducing mitochondrial ROS production.

## *SS peptides inhibit mitochondrial permeability transition*

Mitochondrial ROS are known to contribute to MPT and mitochondrial swelling elicited by high $Ca^{2+}$ and Pi. In turn, mitochondrial $Ca^{2+}$ overload stimulates ROS production. SS-02 and SS-31, by reducing mitochondrial ROS, also effectively reduced mitochondrial swelling in isolated guinea pig cardiac mitochondria induced by high $Ca^{2+}$ and Pi (Fig. 5). Similar findings were obtained with isolated mouse liver mitochondria

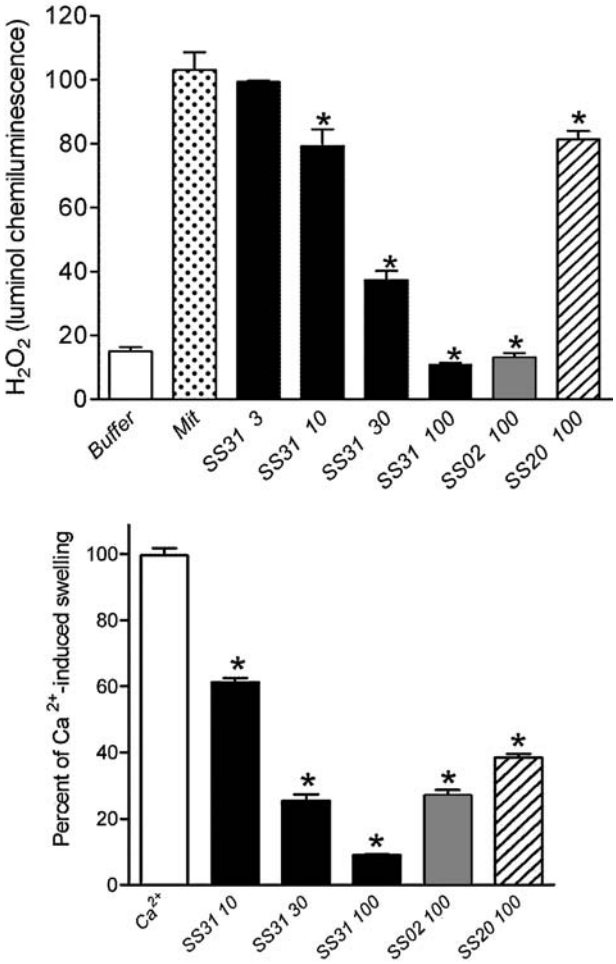


**FIG. 5. SS peptides reduce spontaneous $H_2O_2$ production and inhibit swelling in isolated guinea pig cardiac mitochondria.** Hydrogen peroxide was measured by luminol chemiluminescence in the presence of horseradish peroxidase (HRP). Mitochondrial swelling was induced by high $Ca^{2+}$ and Pi and measured by absorbance at 540 nm. All three SS peptides were able to inhibit Ca2+/Pi induced mitochondrial swelling. $*p < 0.05$.

(120). The inhibition of $Ca^{2+}$ and Pi-induced MPT by SS peptides prevented cytochrome c release into the cytosol (120), and this is relevant to the ability of these SS peptides in ischemia protection (see later section on Ischemia—reperfusion injury). Interestingly, SS20 was also able to inhibit mitochondrial swelling induced by high $Ca^{2+}$ and Pi. Although SS20 does have some minimal ability to reduce mitochondrial ROS production, it is unlikely that the effect would be sufficient to inhibit MPT. Furthermore, other antioxidants known to inhibit mitochondrial ROS, such as trolox and *N*-acetylcysteine, are unable to prevent $Ca^{2+}$-induced mitochondrial swelling (99). It is not known whether the $TPP^+$-conjugated antioxidants, such as MitoQ, are able to inhibit MPT, but higher concentrations of MitoQ were reported to induce mitochondrial depolarization (41, 96).

In addition to MPT induced by $Ca^{2+}$ overload, SS peptides can inhibit MPT elicited by inhibitors of the mitochondrial electron transport chain. $MPP^+$ (1-methyl-4-phenylpyridium), the active metabolite of MPTP (1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine), is known to inhibit mitochondrial complex I activity, reduce $O_2$ consumption, increase ROS production, and cause mitochondrial depolarization and swelling (13). $MPP^+$-induced inhibition of mitochondrial $O_2$ consumption was significantly attenuated by addition of either SS-31 (50 $\mu M$) or SS-20 (100 $\mu M$) (K.S. Zhao and H.H. Szeto, unpublished results). In addition, SS-31 and SS-20 prevented $MPP^+$-induced swelling in isolated mouse liver mitochondria (K.S. Zhao and H.H. Szeto, unpublished results). Since SS-20 does not scavenge ROS, these findings suggest that SS20 and the other SS peptides directly inhibit MPT. This idea is further supported by the finding that SS20 can protect cell viability during ischemia (see later section on Ischemia—reperfusion injury)

## *SS peptides protect mitochondrial function and inhibit cell death in cultured cells*

By targeting and concentrating >1000-fold in the mitochondrial inner membrane, the tyrosine-containing SS peptides (SS-02 and SS-31) are extremely potent in preventing oxidative cell death. Exposure of neuroblastoma $N_2A$ cells to $H_2O_2$ resulted in necrotic cell death that was completely prevented by 1 $\mu M$ SS-31 (75). SS-31 was also very effective in preventing necrotic cell death in G93A Cu,Zn SOD1-transfected $N_2A$ cells. About 20% of cases of familial amyotrophic lateral sclerosis carry this mutation (83), and motor neuron-like cells carrying this mutation have increased ROS production, increased lipid peroxidation, increased cytochrome c release, and reduced cell viability (34). Furthermore, G93A SOD1 mutant cells are more sensitive to the cytotoxicity of $H_2O_2$. This enhanced sensitivity is apparently due to increased free radical generation in response to $H_2O_2$ rather than abnormal SOD activity (114). SS-31 was able to protect G93A Cu,Zn SOD1-transfected cells from $H_2O_2$-induced cell death at 1 $\mu M$ (75). It was apparent from this study that doses lower than 1 $\mu M$ of SS-31 would have been effective in protecting against oxidative cell damage. In contrast, an earlier study reported increased cell viability in G93A SOD1 mutant-expressing cells with 20 m*M* of *N*-acetylcysteine or 25 m*M* of DMPO, a highly effective spin trapping agent (55). At the present time, SS-31 is by far the most potent cytoprotective agent against oxidative stress. Mice overex-

pressing G93A SOD1 mutation show motor neuron disease similar to amyotrophic lateral sclerosis. Daily treatment of these transgenic mice with SS-31 (5 mg/kg, i.p.) delayed the onset of neurological signs and prolonged survival (75). Histopathologic studies revealed significant protection of spinal cord motor neurons in mice receiving daily SS-31 (75). A much larger dose of DMPO (5 mg/mouse, i.p.) was required to delay neurological symptoms and prolong survival in these mice (55), while vitamin E only delayed disease onset without increasing survival time (33).

*Tert*-butylhydroperoxide (*t*BHP) is a membrane-permeant prooxidant compound that can induce cell death via apoptosis or necrosis. SS-31 was extraordinarily potent in reducing intracellular ROS and preventing apoptosis in neuronal cells after tBHP treatment, with $EC_{50}$ in the ~0.1 n*M* (118) In contrast, most antioxidants require at least 100 $\mu M$ to reduce oxidative cell death (4, 68). MitoQ was able to block $H_2O_2$-induced apoptosis at 1 $\mu M$, but >10 $\mu M$ caused cytotoxicity (41). The remarkable potency of SS-31 in whole cells can be accounted for by its >1000-fold concentration in the inner mitochondrial membrane. Thus local concentration of SS-31on the inner mitochondrial membrane may be >1 $\mu M$ when extracellular concentration is only 1 n*M*. In addition to its extraordinary potency, SS-31 is not cytotoxic in normal cells even at 1 m*M*, resulting in a therapeutic window in excess of a millionfold.

*t*BHP-induced apoptosis appears to be triggered by MPT, and trifluoperazine (5 $\mu M$), an inhibitor of MPT, was able to reduce cell death in hepatocytes treated with *t*BHP (68, 78). Treatment of neuroblastoma $N_2A$ cells with *t*BHP resulted in mitochondrial depolarization and decreased mitochondrial function (118). SS-31 dose-dependently inhibited *t*BHP-induced mitochondrial depolarization and preserved mitochondrial function at 1 n*M* (118). The much greater potency of SS-31 compared to trifluoperazine may be partly due to the selective partitioning of SS-31 to the inner mitochondrial membrane, and partly due to its antioxidant properties. It has been shown that deferoxamine (0.6 m*M*), an inhibitor of iron-catalyzed hydroxyl radical scavenger, is more effective than trifluoperazine in preventing *t*BHP-induced cell death, suggesting that ROS contribute significantly to the induction of MPT by *t*BHP. It is clear that SS-31 is the most potent compound reported that can inhibit cell death caused by *t*BHP. The prevention of MPT by SS-31 will minimize MPT-induced ROS accumulation and reduce further oxidative damage on mitochondria. By preventing MPT, SS-31 was able to prevent phosphatidylserine translocation, caspase-9 activation, and nuclei condensation associated with apoptotic cell death (118).

Other studies have shown that SS peptides can prevent cell death induced by inhibitors of the electron transport chain. In one study, SS-02 prevented mitochondrial depolarization induced by 3-nitroprionic acid in Caco-2 cells (120). 3-Nitroprionic acid is an irreversible inhibitor of the complex II enzyme succinate dehydrogenase. In another study, SS-31 and SS-20 dose-dependently prevented cell death elicited by $MPP^+$ in SN4741 dopamine cells. $MPP^+$ is known to inhibit complex I activity, reduce $O_2$ consumption, increase ROS production, and leads to mitochondrial depolarization and swelling (13). Studies with isolated brain mitochondria showed that SS-31 and SS-20 (50–100 $\mu M$) can both prevent the $MPP^+$-induced reduction in $O_2$ consumption and mitochondrial swelling (K.S. Zhao and H.H. Szeto, unpublished results). However, a significant reduction in apoptotic cells caused by $MPP^+$ was observed with just 1 n*M* of either SS-31 or SS-20. The large discrepancy between the effective peptide concentrations required for isolated mitochondria studies versus whole cell studies supports the tremendous ability of these peptides to concentrate in the inner mitochondrial membrane. Thus the targeting of SS-31 to inner mitochondrial membrane allows the use of a very low concentration of SS-31 for selective scavenging of mitochondrial $H_2O_2$ without affecting cytoplasmic or extracellular $H_2O_2$. For example, dopamine causes cell cycle arrest in proliferating lymphocytes via extracellular $H_2O_2$ (60). While this action of dopamine was readily inhibited by extracellular catalase, it was not blocked by SS-31 at concentrations that have been shown to prevent mitochondria-mediated apoptosis (60).

## ROLE OF MITOCHONDRIAL DYSFUNCTION AND OXIDATIVE DAMAGE IN ISCHEMIA—REPERFUSION INJURY

Ischemia is associated with lack of oxygen and substrate delivery to the tissue and results in impaired energy metabolism that, if prolonged, can result in cell death. Myocardial ischemia and cerebral ischemia are two leading causes of death in the western world. Persistent ischemia will lead to cell death. With brief periods of ischemia, the depressed cellular energy state can be fully reversed on reperfusion, and cells remain viable and functional. Coronary reperfusion utilizing thrombolytics, coronary angioplasty, or by-pass surgery can, if performed in a timely fashion, partially rescue the ischemic myocardium and limit the size of the infarct. However, reperfusion itself, especially after prolonged ischemia, may also lead to increased cell death, a phenomenon known as "reperfusion injury." Reperfusion injury is also encountered in organ transplantation after the organ has been subjected to prolonged ischemic storage.

Progressive ischemia leads to inhibition of several components of the mitochondrial electron transport chain, including complex I, complex V, and the adenine nucleotide translocator, and decrease in ATP production [for review, see (51)] . In addition, ischemia decreases cardiolipin and cytochrome c content in cardiac mitochondria (50, 52), both of which will inhibit cytochrome c oxidase activity (115). There is also evidence to support ROS generation during ischemia (53), and this leads to cardiolipin peroxidation and further inhibition of cytochrome c oxidase activity (50) (Fig. 6).

With the decrease in mitochondrial ATP production, the cardiac cell strives to maintain ATP production by increasing glycolysis. However, glycolysis is much less efficient in ATP generation, and during ischemia, the $F_0F_1$ ATPase begins to actively hydrolyze ATP rather than synthesize ATP, resulting in a dramatic reduction in ATP and corresponding increase in Pi (Fig. 6). The activation of glycolysis also leads to accumulation of lactic acid and decrease in intracellular pH. Intracellular pH can decrease to <6.0 after only 10 min of ischemia. In an attempt to correct this $H^+$ accumulation, the $Na^+/H^+$ exchanger results in increased intracellular $Na^+$ (45). The inhibi-

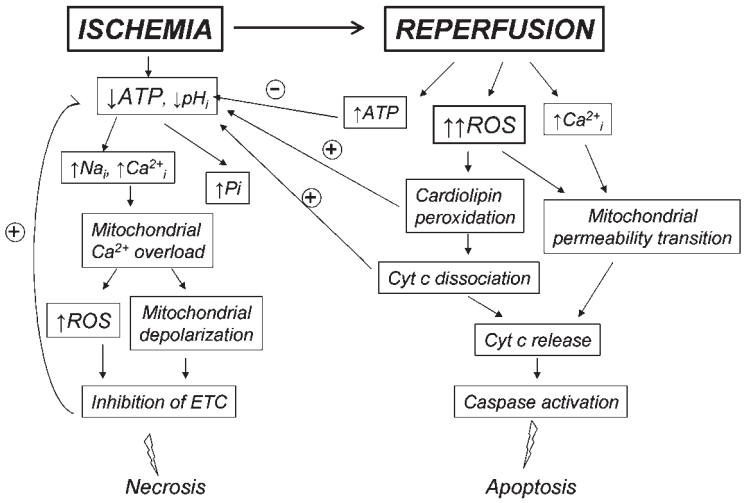


**FIG. 6. A scheme showing mitochondrial dysfunction and oxidative damage during cardiac ischemia—reperfusion.** Progressive ischemia leads to inhibition of the mitochondrial electron transport chain (ETC) and decrease in ATP production. The cardiac cell strives to maintain ATP production by increasing glycolysis, resulting in further reduction in ATP, increase in Pi, and decreased intracellular pH. Activation of the $Na^+/H^+$ exchanger, and inhibition of $Na^+/K^+$ ATPase, results in increased intracellular $Na^+$. Intracellular $Ca^{2+}$ becomes elevated via the $Na^+/Ca^{2+}$ exchanger, resulting in mitochondrial $Ca^{2+}$ overload and mitochondrial depolarization. In addition, ROS production and cardiolipin peroxidation further contributes to loss of inner membrane integrity, inhibition of the ETC, and mitochondrial depolarization. Restoration of blood flow will help to restore ATP levels, but the damaged mitochondria generate enormous amounts of ROS during reperfusion, leading to cardiolipin peroxidation which would further inhibit oxidative phosphorylation and promote mitochondrial permeability transition (MPT). Opening of the MPT pore results in cytochrome c release into the cytoplasm, activation of the caspase cascade, and apoptosis.

tion of $Na^+/K^+$ ATPase further increases intracellular $Na^+$ and results in increased intracellular $Ca^{2+}$ via the $Na^+/Ca^{2+}$ exchanger (101). Increased cytosolic $Ca^{2+}$ leads to mitochondrial $Ca^{2+}$ overload driven by the potential gradient across the inner membrane and via the $Ca^{2+}$ uniporter, resulting in mitochondrial depolarization (25). In addition, cardiolipin peroxidation further contributes to loss of inner membrane integrity and mitochondrial depolarization (76).

Restoration of blood flow will help to limit infarct size, but recovery of mitochondrial function depends on the duration of ischemia. With prolonged ischemia, there is evidence that the rate of oxidative phosphorylation continues to deteriorate despite reperfusion, suggesting that additional mitochondrial dysfunction can take place (51). The damaged mitochondria generate enormous amounts of ROS during reperfusion, and there is an excess of hydroxyl radicals as a result of iron overload following ischemia. This will lead to cardiolipin peroxidation and further inhibit oxidative phosphorylation and promote MPT. The amount of ROS generation upon reperfusion is dependent on the extent of mitochondrial injury suffered during ischemia.

Although the conditions of excess Pi, elevated mitochondrial $Ca^{2+}$, and ROS all increase the probability of MPT pore opening during ischemia, most evidence suggest that the pore remains closed during ischemia and does not open until reperfusion takes place (31). The increase in mitochondrial $Ca^{2+}$ flux with the restoration of mitochondrial respiration during reperfusion promotes the opening of the MPT pore (35). It has been proposed that ischemia is the MPT priming period, while reperfusion is the MPT trigger period, and the duration of ischemia would determine the susceptibility of myocytes to MPT upon reperfusion (108). Unless the pore closes rapidly, the cell would not be able to survive, and the cell dies by necrosis as a result of loss of plasma membrane integrity. If the pores close quickly, maintenance of ATP will switch from necrosis to apoptosis. In addition, the reactivation of mitochondrial respiration upon post-ischemic reperfusion generates a burst of superoxide that cannot be readily eliminated. ROS production is also exacerbated by mitochondrial $Ca^{2+}$ that accumulates during reperfusion. This oxidative burst during reperfusion serves to promote $Ca^{2+}$-induced MPT and apoptosis, adding to the ischemic damage.

The mitochondrial permeability transition pore and ROS are widely considered the most promising targets for cardioprotection in ischemia—reperfusion injury. But Fig. 6 clearly shows that inhibition of cardiolipin peroxidation, maintenance of electron transport chain function, and preserving mitochondrial potential can all contribute to cardioprotection. Because of the multiple pathways by which the SS peptides may limit ischemia—reperfusion damage, these peptides have been studied in several animal models of ischemia—reperfusion injury (see below).

## EVALUATION OF SS PEPTIDES IN MODELS OF ISCHEMIA—REPERFUSION INJURY

### *SS peptides protect contractile function in myocardial ischemia—reperfusion*—ex vivo *studies*

The SS peptides were first evaluated in ischemia—reperfusion injury in *ex vivo* guinea pig hearts undergoing retrograde perfusion in a Langendorff apparatus. The isolated hearts were subjected to 30 min global ischemia, followed by 90 min reperfusion. The SS peptides (1 n*M*) were introduced either upon reperfusion (Fig. 7) or before ischemia and throughout reperfusion (Fig. 8). Despite restoration of flow after ischemia, the buffer-treated hearts showed progressive deterioration of contractile force during the reperfusion period, reflecting a stunned myocardium. Contractile force at 10 min and 90 min after onset of reperfusion was significantly improved if SS-31 was

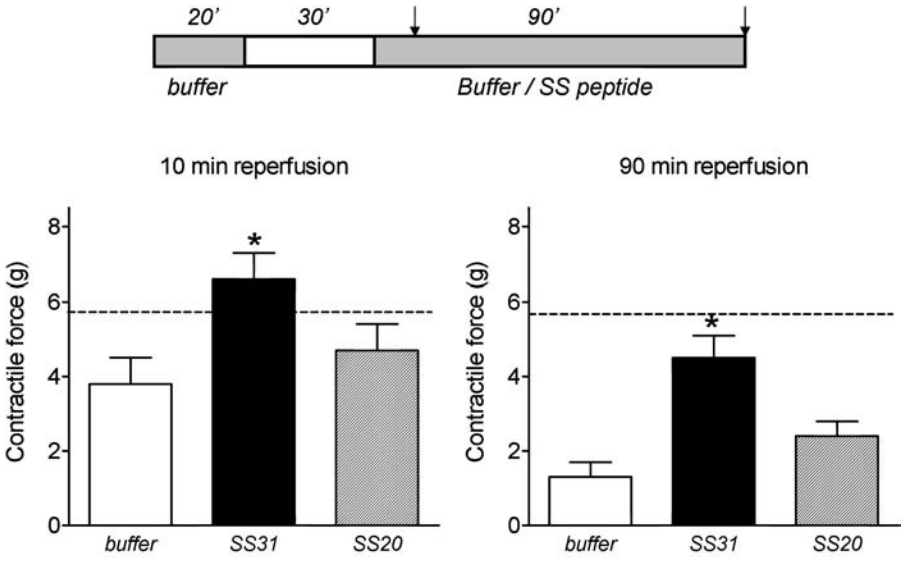


**FIG. 7. Post-ischemic treatment with SS-31 improves myocardial contractile activity after global ischemia in the *ex vivo* heart.** Isolated guinea pig hearts were perfused in a retrograde manner with Krebs—Henseleit buffer in a Langendorff apparatus. After a 20 min stabilization period, global ischemia was induced for 30 min, followed by 90 min reperfusion with or without 1 n*M* of SS-31 or SS-20. Post-ischemic treatment with SS-31 significantly improved contractile force, especially in the late reperfusion period. SS-20 was not effective. The *dashed line* shows mean contractile force prior to global ischemia. $*p < 0.05$ compared to buffer alone.

given upon reperfusion only (Fig. 7). SS-20, on the other hand, offered no protection if given after ischemia. Although a number of pharmacological agents have been shown to improve cardiac function if given prior to ischemia (preconditioning), SS-31 is one of the very few agents that can improve contractile force even when it is given upon reperfusion. A previous report showed that SS-02 was also able to prevent myocardial stunning in the *ex vivo* heart when given upon reperfusion (113), and this was later confirmed *in vivo* (see below) (97).

SS-31 and SS-02 are currently the most potent (n*M*) cardioprotective agents that can be used to reduce myocardial stunning without pre-ischemic treatment. These studies lend support to oxidative free radicals being the major cause of myocardial stunning (9) because SS-20 was ineffective when given upon reperfusion. By selectively partitioning to the inner mitochondrial membrane, SS-31 and SS-02 can scavenge mitochondrial ROS and inhibit cardiolipin peroxidation, thus inhibiting the onset of MPT. Opening of the MPT pore in the early phase of reperfusion is believed to underlie contractile dysfunction in the stunned myocardium. Inhibition of MPT pore opening at the onset of reoxygenation with cyclosporin A and sanglifehrin A were reported to improve contractile function in human atrial tissue (91). Recently, a novel SOD-mimetic MPT inhibitor (HO-3538) was reported to significantly enhance the recovery of mitochondrial energy metabolism and contractile function (8). The SS peptides are at least 100-fold more potent than these other agents in inhibiting MPT during ischemia—reperfusion.

Surprisingly, even though SS-20 was ineffective during reperfusion, pre-ischemic treatment with SS-20 significantly

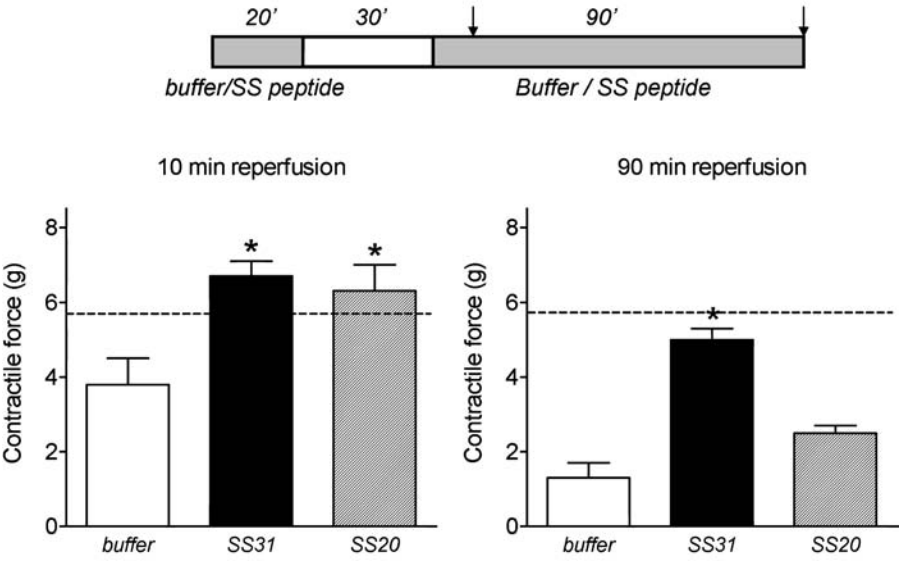


**FIG. 8. Pre-ischemic treatment with SS peptides improves myocardial contractile activity in the *ex vivo* heart.** Isolated guinea pig hearts were perfused in a retrograde manner with Krebs—Henseleit buffer alone, or buffer containing either 1 n*M* SS-31 or SS-20, in a Langendorff apparatus. After a 20 min stabilization period, global ischemia was induced for 30 min, followed by 90 min reperfusion with or without 1 n*M* of SS-31 or SS-20. Pre-ischemic treatment with either SS-31 or SS-20 significantly improved contractile force in the early reperfusion period, but only SS-31 prevented myocardial stunning in the late reperfusion period. The *dashed line* shows mean contractile force prior to global ischemia. $*p < 0.05$ compared to buffer alone.

improved contractile function in early reperfusion (Fig. 8). However, the protective effect of SS-20 was not sustained during late reperfusion, perhaps due to overwhelming ROS production. These studies suggest that the SS peptides may improve mitochondrial function during ischemia. This idea is supported by subsequent *in vivo* studies.

## *SS peptides reduce infarct size in model of LAD occlusion*

The ability of SS peptides to protect against myocardial damage during ischemia was further confirmed in an *in vivo* model where the LAD (left anterior descending coronary artery) was occluded for 60 min in rats. Rats received an intraperitoneal injection of either saline, SS-31 (3 mg/kg) or SS-20 (3 mg/kg) 30 min prior to occlusion of the LAD, and another dose 5 min before onset of reperfusion for another 60 min. Pre-ischemic treatment with either SS-31 or SS-20 significantly improved myocardial ATP content, decreased lipid peroxidation (as measured by 4-hydroxynonenal), reduced infarct size, and prevented ventricular arrhythmias during early reperfusion (Fig. 9) (15). Although both peptides reduced infarct size compared with saline control, interestingly the peptide without scavenging activity (SS-20) preserved the ischemic myocardium even better than the scavenging peptide (SS-31). The study provided some interesting information about SS-20. Even though SS-20 does not scavenge $H_2O_2$, nor does it inhibit lipid peroxidation *in vitro* (120), it was able to reduce lipid peroxidation in the ischemic myocardium. This suggests that SS-20 can reduce mitochondrial ROS production during ischemia—reperfusion, most likely by improving mitochondrial bioenergetics during ischemia. This is consistent with higher ATP content in the ischemia myocardium.

## *SS peptides reduce myocardial stunning in model of LAD occlusion*

In addition to improving mitochondrial bioenergetics during ischemia, a recent study reported that pre-ischemic treatment with SS-02 in a similar model of LAD occlusion in rats reduced reperfusion arrhythmias, shortened the time to left ventricular recovery, with significant improvement in ejection fraction and systolic wall thickening (97) (see Table 1). Thus, the SS peptides not only reduce necrotic cell death (infarct size) in ischemia—reperfusion, they can also reduce myocardial stunning *in vivo*.

## *SS peptides reduce infarct size in model of cerebral ischemia—reperfusion*

SS-31 has been investigated in a mouse model of cerebral ischemia—reperfusion achieved by transient occlusion of the middle cerebral artery for 30 min, followed by reperfusion (71). Oxidative stress significantly reduced glutathione levels in the ipsilateral cortex and striatum. Treating mice with SS-31 (2 mg/kg or 5 mg/kg given intraperitoneally) immediately after reperfusion, and at 6, 24, and 48 h after ischemia significantly reduced glutathione depletion in the cortex and reduced infarct size by 32% and 35%, respectively, in the two dose groups. Focal ischemia is normally associated with a local inflammatory reaction that contributes to tissue damage after the ischemic insult. Treatment with SS-31 also significantly decreased hemispheric swelling. The success of SS-31 in reducing infarct size when given after onset of reperfusion is significant for clinical applications, and is likely enhanced by the its rapid distribution across the blood—brain barrier.

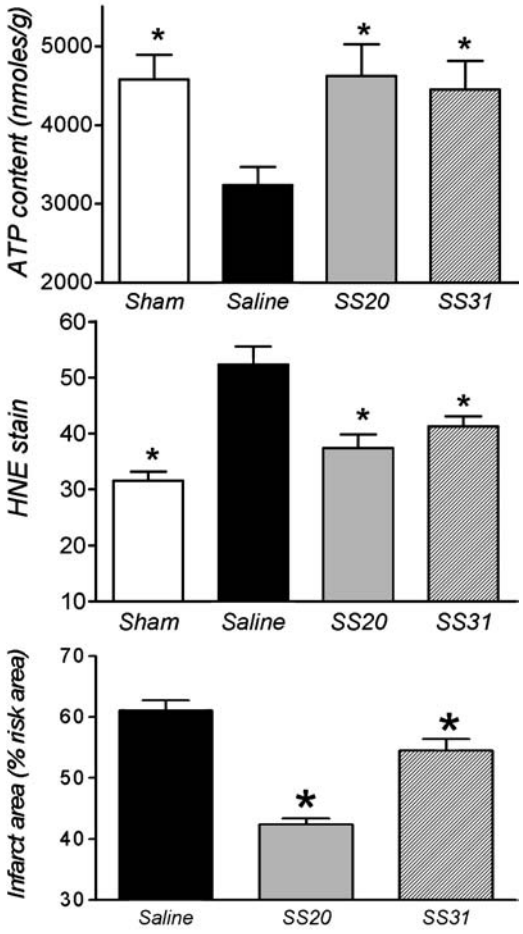


**FIG. 9. SS-31 and SS-20 improved cardiac outcome after transient occlusion of the LAD (left anterior descending coronary artery).** Rats were given either saline, SS-31 (3 mg/kg, ip) or SS-20 (3 mg/kg, ip) 30 min prior to occlusion of the LAD, and another dose 5 min before onset of reperfusion for another 60 min. Pre-ischemic treatment with either SS-31 or SS-20 significantly improved myocardial ATP content, decreased lipid peroxidation (as measured by 4-hydroxynonenal (HNE)), reduced infarct size, and prevented ventricular arrhythmias during early reperfusion. $*p < 0.05$ compared to saline control.

## *SS peptides preserve coronary flow in hearts after prolonged cold ischemic storage and warm reperfusion*

Ischemia—reperfusion injury is also a necessary consequence of all organ and cell transplantation. Prolonged cold ischemic storage results in mitochondrial damage, and transplantation of the ischemic organ is met with oxidative damage. Cardiac transplantation is hindered by donor shortage and preservation time. Cold ischemic storage of hearts for transplantation is currently limited to 4–6 h, with longer storage time

associated with compromised short-term and long-term outcomes. Endothelial injury is now recognized as the most important cause of poor outcome after cardiac transplantation (26). The SS peptides have been investigated in a model of prolonged cold ischemic storage—warm reperfusion of guinea pig hearts. Isolated hearts were perfused in a Langendorff apparatus with a cardioplegic solution alone, or cardioplegic solution containing SS-31 (1 n*M*) or SS-20 (100 n*M*), for 3 min, and then stored in the same solution at 4ºC for 18 h. After the storage time, the hearts were re-mounted in the Langendorff apparatus and perfused with Krebs—Henseleit buffer (34ºC) for 90 min. Coronary flow was reduced 70% at the end of warm reperfusion in hearts stored in the cardioplegic solution alone compared to pre-ischemic flow. In contrast, hearts stored with either SS-31 or SS20 in the cardioplegic solution had significantly better coronary flow, with only 10–20% reduction compared to pre-ischemic values. The compromised coronary flow in the heart stored for prolonged time in cardioplegic solution alone was due to extensive apoptosis of the endothelial cells in the coronary vessels (Fig. 10). In contrast, endothelial apoptosis was absent in the hearts stored with SS-31 or SS-20. Endothelial cell apoptosis is a significant problem in cold ischemic storage of other organs as well, and caspase inhibition has been reported to improve ischemia—reperfusion injury after lung transplantation. These results suggest that the SS peptides, by preventing endothelial apoptosis, may allow much longer cold ischemic storage time and significantly increase organ availability for transplantation.

### *SS peptides preserve pancreatic islet cells during isolation and improves transplantation outcome*

Stressors present during pancreas procurement, preservation, isolation, and following transplantation contribute to islet cell demise and poor transplant outcome. The islet isolation procedure disrupts islet cells from their extracellular matrix via an enzymatic and mechanical process and subjects the islets to cytokine, oxidative, and ischemic stress (1, 10). Improved glucose control was observed in a marginal islet mass model with islets from mice overexpressing glutathione peroxidase, MnSOD, and CuZnSOD, suggesting that oxidative stress plays a major role in islet viability (65). Supplementation of isolation buffers with 1 n*M* SS-31 significantly increased islet yield and mitochondrial potential of the islet cells, and reduced percent of apoptotic cells from by 50% (102). In a marginal mass transplantation model in mice, 5 of 10 recipients of islets isolated in the presence of 1 n*M* SS-31 remained normoglycemic post transplantation, whereas all of the animals who received control islets remained diabetic (102). These findings suggest that SS-31 can improve islet survival during the isolation process and also improve graft survival during the immediate ischemic phase after transplantation. SS-31 may serve to increase the pool of eligible organ donors for treatment of type I diabetes.

## THERAPEUTIC POTENTIAL OF SS PEPTIDES

The *in vivo* animal studies described above suggest that the SS peptides may be beneficial against ischemia—reperfusion injury. However, the pharmaceutical development of peptide drugs has been fraught with difficulties. The disadvantages of peptide molecules include solubility challenges, lack of stability, difficulty with transport across membranes (especially blood—brain barrier), rapid clearance from body, and low oral bioavailability. Surprisingly, preliminary studies have shown that the SS peptides have excellent pharmacokinetic properties in several animal species, making them extremely feasible for pharmaceutical development. Furthermore, loss of cell viability was only observed at concentrations that are >1000-fold higher than concentrations required to prevent oxidative cell death, and no apparent toxicity has been observed in animal studies even after chronic dosing.

### *Pharmacokinetic studies of SS peptides in animal models*

All three SS peptides illustrated in Fig. 2 bear 3+ net charge at physiologic pH and are therefore readily soluble in water. Stability studies have shown that these tetrapeptides are stable in aqueous solution for as at least 6 months, even when stored at 37ºC. Because of their solubility in water, these peptides can be administered via many routes, including intravenous, subcutaneous, intraperitoneal, intrathecal, and intracerebroventricular injections (15, 16, 75, 95, 97, 102, 117).

*In vivo* pharmacokinetic studies were made possible with the development of highly specific and sensitive liquid chromatography—mass spectrometry methods (32, 106, 107). Early pharmacokinetic studies of SS-02 were carried out in sheep utilizing continuous intravenous infusion (100). The calculated pharmacokinetic parameters revealed an apparent volume of distribution (~60 ml/kg) and an elimination half-life of ~1.8 hours. The very small apparent volume of distribution is consistent with the highly polar character of these 3+ charge peptides, suggesting very little accumulation of these peptides in adipose tissue. Yet, SS-02 is rapidly distributed to the brain and other highly perfused organs including lung, heart, and kidneys. Following an intravenous administration of [$^3$H]SS-02 to mice, radioactivity peaked in the brain and other organs before 5 min. The rapid distribution of SS-02 across the blood—brain barrier was unexpected for a polar compound, but is consistent with its rapid transcellular uptake across an epithelial cell monolayer (119). In addition, SS-02 has high affinity for opioid receptors (116) and onset of analgesia was very rapid after subcutaneous administration in mice, with peak analgesic effect observed between 30–45 min (67, 117). Extensive brain uptake of SS-02 is supported by the finding that SS-02 was 36 times more potent than morphine after subcutaneous administration (117). In contrast, other synthetic opioid peptides are generally not potent

Table 1. Effects of SS-02 on Left Ventricular Function after Transient LAD Occlusion

| | *Saline* | *SS-02* |
|---|---|---|
| Time to recovery (min) | 10.5 ± 2.2 | 4.4 ± 2.2* |
| Fractional shortening (%) | 32.1 ± 9.1 | 45.7 ± 2.4† |
| Ejection fraction (%) | 32.3 ± 13.7 | 48.9 ± 10.9† |
| Systolic wall thickening (%) | 33.5 ± 7.7 | 50.5 ± 8.4* |

*$p < 0.01$.
†$p < 0.05$.

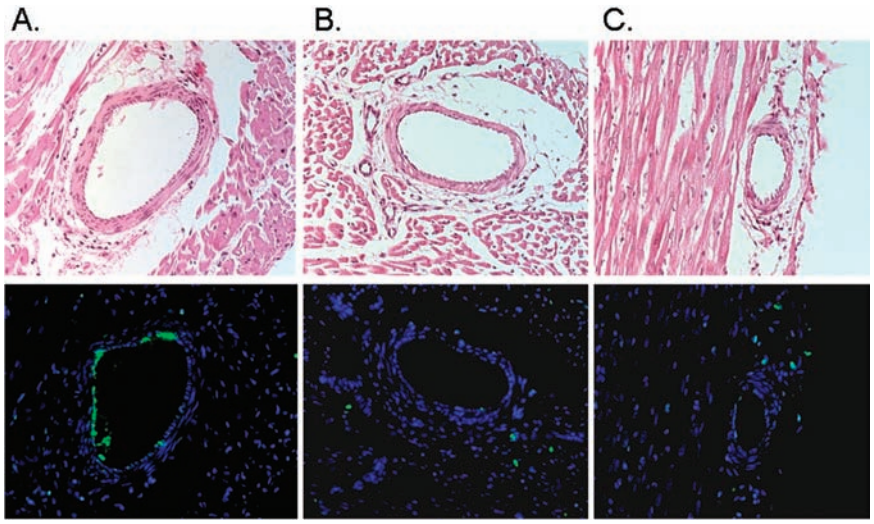


**FIG. 10. SS peptides prevent endothelial apoptosis in hearts subjected to prolonged cold ischemic storage and warm reperfusion.** Isolated guinea pig hearts were perfused in a Langendorff apparatus with a cardioplegic solution alone, or cardioplegic solution containing SS-31 (1 n*M*) or SS-20 (1 or 100 n*M*) for 3 min, and then stored in the same solution at 4°C for 18 hours. After the storage time, the hearts were re-mounted in the Langendorff apparatus and perfused with Krebs—Henseleit buffer (34°C) for 90 min. Hearts were then set in paraffin blocks, sectioned, and stained for apoptotic cells using the TUNEL method with fluorescent secondary antibody. Extensive TUNEL-positive endothelial cells (*green*) were found in hearts stored in buffer (**A**) while storage with either SS-31 (**B**) or SS-20 (**C**) prevented apoptosis.

when given systemically. Thus the aromatic—cationic nature of the SS peptides allows them to readily cross the blood—brain barrier.

The relatively long elimination half-life of SS-02 is highly unusual for a peptide. The SS peptides were designed to minimize enzymatic degradation, with the incorporation of a *D*-amino acid either in the first (SS-31) or second residue (SS-02), and amidation of the carboxy terminus. These tetrapeptides do not undergo degradation when incubated in whole blood for 2 hours (100). Being such polar molecules, the SS peptides might be expected to undergo renal clearance. Surprisingly, the plasma clearance of SS-02 from blood (23 ml/kg/h) was found to be almost 10-fold slower than creatinine clearance (~210 ml/kg/h), suggesting substantial tubular reabsorption of this peptide, most likely via PEPT2, the peptide transporter present on proximal tubular cells of the kidney (100).

The pharmacokinetics of SS-02, SS-31, and SS-20 have been confirmed in rats following intravenous bolus administration and their elimination half-lives ranged from 30 to 40 min. The systemic absorption of these peptides following intraperitoneal administration is rapid, with peak plasma levels observed before 20 min. Despite the relatively short half-lives, a single subcutaneous dose of SS-02 (1 mg/kg) was found to produce significant analgesic response for more than 12 h in mice (117). Similarly, once a day dosing with intraperitoneal SS-31 (5 mg/kg) was sufficient to protect spinal cord motor neurons in a transgenic mouse model of amyotropic lateral sclerosis (75). Significant improvement in pancreatic islet transplantation was also achieved with once a day dosing with SS-31 (102). Because these peptides are stable in aqueous solution at 37°C, they can also be delivered using mini-osmotic pumps for sustained subcutaneous delivery over 2–4 weeks in mice and rats.

Although peptides are generally considered to be poor candidates for oral administration, available data suggest that these SS peptides may have reasonable oral bioavailability. Oral absorption can be predicted using Caco-2 cells (human intestinal epithelial cell) grown in a monolayer. The minimum $P_{app}$ (apparent permeability coefficient) required to anticipate 100% absorption in humans has been estimated between $1 \times 10^{-6}$ and $6 \times 10^{-5}$ cm/s (30, 84). With a calculated $P_{app}$ of $1.24 \times 10^{-5}$ cm/s for SS-02 (119), we may anticipate reasonably good oral absorption for these peptides. Furthermore, oral administration of SS-02 produced dose-dependent analgesia in mice (Szeto, unpublished results), demonstrating that these SS peptides are orally active.

### *Cytotoxicity studies in cultured cells*

The SS peptides have been found to be relatively free of cytotoxicity in cultured cells. SS-02 and SS-31 have been shown to be effective in preventing cell death induced by oxidative damage in the concentration range of 0.1–100 n*M* (118, 120). When given alone, these peptides had no effect on cell viability in epithelial cells (Hela, Caco-2, HKC-8, MDCK) or neuroblastoma cells ($N_2$A, SH-SY5Y) even at 1 m*M*.

## In vivo *toxicity*

The SS peptides have been studied extensively in animal models. Because of its high affinity for opioid receptors, SS-02 can cause constipation (67) and respiratory depression (94) similar to morphine. In contrast, SS-31 and SS-20 have negligible affinity for opioid receptors and are therefore devoid of opioid receptor-mediated effects at therapeutic doses. SS-31 has been administered daily (5 mg/kg) for up to 5 months in a mouse model of amyotrophic lateral sclerosis and no adverse effects were observed while treatment was actually associated with significant protection of spinal cord motor neurons, delayed onset of neurological deficits, and increased survival time (75). In other studies, repeated dosing with higher doses of SS-31 (up to 20 mg/kg) for 14 days in mice did not result in any apparent toxicity. While available studies suggest that these SS peptides appear to be free of toxic side effects, formal toxicological studies will need to be conducted to determine the relative

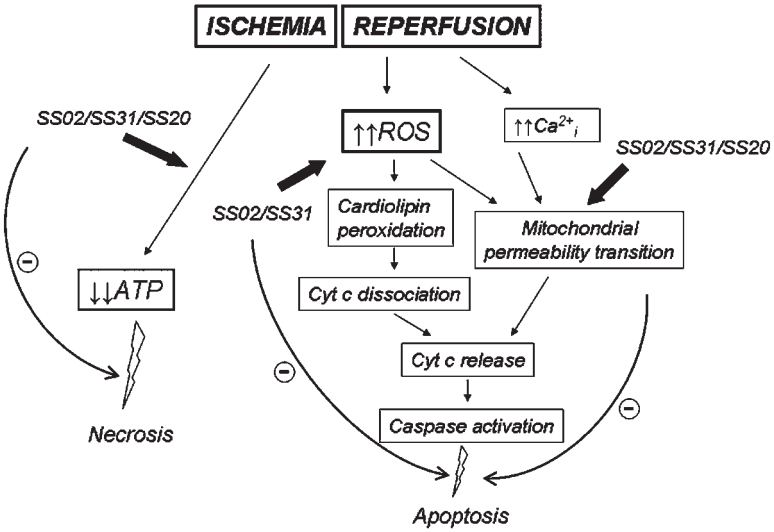


**FIG. 11. Proposed sites and mechanisms of action for the SS peptides in protecting against ischemia—reperfusion injury.** Pre-ischemic treatment with any of the three peptides (SS-02, SS-31, or SS-20) protects the mitochondrial ETC and helps maintain ATP levels during ischemia. All three peptides can inhibit mitochondrial permeability transition induced by $Ca^{2+}$/Pi. Upon reperfusion, there is a massive burst of ROS activity, and only the scavenging peptides (SS-02 and SS-31) can effectively minimize mitochondrial permeability transition.

safety of these novel compounds for pharmaceutical development.

## SUMMARY

It is now recognized that oxidative injury and mitochondrial dysfunction are responsible for many clinical disorders with unmet needs. Mitochondrial dysfunction can lead to cell death by apoptosis and necrosis. As mitochondria are the major source of intracellular ROS, and mitochondria are also the primary target for ROS, the ideal drug therapy needs to be targeted to mitochondria. The mitochondria-targeted cytoprotective peptides described in this review represent a novel class of mitoprotective agents that are selectively targeted to and concentrate in mitochondria. Extensive data from in vivo animal studies support their clinical utility in ischemia—reperfusion injury. Figure 11 summarizes the mechanisms by which the SS peptides can help minimize ischemia—reperfusion injury. These peptides can be divided into two major groups. Those containing tyrosine or modified tyrosine residues can scavenge ROS, and are particularly effective in disorders associated with significant ROS generation, such as reperfusion injury in post-ischemic tissues. Those that do not have scavenging capability are still highly effective in preventing ischemic injury and can be used for minimizing ischemia—reperfusion injury during angioplasty, coronary bypass surgery, cardiac surgery, and organ transplantation. Animal studies to date indicate that these novel mitochondria-targeted peptides have excellent pharmacokinetic properties and are relatively free of toxicity, suggesting that they may have enormous therapeutic potential.

## ACKNOWLEDGMENTS

The author is grateful to the many collaborators who contributed to the work presented in this review. Majority of the work presented in this review was supported by NIDA (PO1 DA08924), NINDS (R21 NS48295), and NIDDK (R01 DK073595). The SS peptides were developed in collaboration with Dr. Peter W. Schiller of the Clinical Research Institute of Montreal, Montreal, Quebec, Canada. All biochemical, cellular, and *ex vivo* studies mentioned in this article were carried out by Drs. Kesheng Zhao, Guomin Zhao, Dunli Wu, and Yi Soong (Department of Pharmacology, Weill Medical College of Cornell University). Special thanks to Drs. Mun Hong, Wooyuk Song, Janghyun Cho and Kyungheon Won (Department of Internal Medicine, Weill Medical College of Cornell University) for the *in vivo* studies with SS peptides in myocardial ischemia—reperfusion; Dr. Sunghee Cho (Burke Research Institute, White Plains, NY, and Department of Neurology/Neuroscience, Weill Medical College of Cornell University) for the studies of SS-31 in cerebral ischemia—reperfusion; and Drs. Dolca A. Thomas and Manikkam Suthanthiran (Departments of Medicine and Transplantation Medicine, Weill Medical College of Cornell University) for the pancreatic islet transplantation studies.

## ABBREVIATIONS

ANT, adenine nucleotide translocator; $CO_3^{\cdot -}$, carbonate radical anion; CoQ, coenzyme Q; Dmt, 2′,6′-dimethyltyrosine; ETC, electron transport chain; FCCP, (carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone); LAD, left anterior descending coronary artery; MOMP, mitochondrial outer membrane permeability; $MPP^+$, 1-methyl-4-phenylpyridium; MPT, mitochondrial permeability transition; MPTP, 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine; NAC, *N*-acetylcysteine; $O_2^{\cdot -}$, superoxide anion; $OH^{\cdot}$, hydroxyl radical; $ONOO^-$, peroxynitrite; PARP, poly-(ADP-ribose)-polymerase; ROS, reactive oxygen species; SOD, superoxide dismutase; SS peptides, Szeto—Schiller peptides; SS-02, Dmt-D-Arg-Phe-Lys-$NH_2$; SS-20, Phe-D-Arg-Phe-Lys-$NH_2$; SS-31, D-Arg-Dmt-Lys-Phe-$NH_2$; TEMPOL, 2,2,6,6-tetramethylpiperidine-1-oxyl; *t*BHP, *Tert*-butylhydroperoxide; TCA, tricarboxylic acid; $TPP^+$, triphenylalkylphosphonium cation; VDAC, voltage-dependent anion channel.

## 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.

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Date of first submission to ARS Central, August 29, 2007; date of final revised submission, September 9, 2007; date of acceptance, September 10, 2007.