CALL FOR PAPERS | Novel Therapeutics in Renal Diseases

Improving mitochondrial bioenergetics under ischemic conditions increases warm ischemia tolerance in the kidney

Hazel H. Szeto, Shaoyi Liu, Yi Soong, and Alexander V. Birk
Research Program in Mitochondrial Therapeutics, Department of Pharmacology, Joan and Sanford I. Weill Medical College of Cornell University, New York, New York

Submitted 27 June 2014; accepted in final form 20 October 2014

Szeto HH, Liu S, Soong Y, Birk AV. Improving mitochondrial bioenergetics under ischemic conditions increases warm ischemia tolerance in the kidney. Am J Physiol Renal Physiol 308: F11–F21, 2015. First published October 22, 2014; doi:10.1152/ajprenal.00366.2014.—Ischemia time during partial nephrectomy is strongly associated with acute and chronic renal injury. ATP depletion during warm ischemia inhibits ATP-dependent processes, resulting in cell swelling, cytoskeletal breakdown, and cell death. The duration of ischemia tolerated by the kidney depends on the amount of ATP that can be produced with residual substrates and oxygen in the tissue to sustain cell function. We previously reported that the rat can tolerate 30-min ischemia quite well but 45-min ischemia results in acute kidney injury and progressive interstitial fibrosis. Here, we report that pretreatment with SS-20 30 min before warm ischemia in the rat increased ischemia tolerance from 30 to 45 min. Histological examination of kidney tissues revealed that SS-20 reduced cytoskeletal breakdown and cell swelling after 45-min ischemia. Electron microscopy showed that SS-20 reduced mitochondrial matrix swelling and preserved cristae membranes, suggesting that SS-20 enhanced mitochondrial ATP synthesis under ischemic conditions. Studies with isolated kidney mitochondria showed dramatic reduction in state 3 respiration and respiratory control ratio after 45-min ischemia, and this was significantly improved by SS-20 treatment. These results suggest that SS-20 increases efficiency of the electron transport chain and improves coupling of oxidative phosphorylation. SS-20 treatment after ischemia also significantly reduced interstitial fibrosis. These new findings reveal that enhancing mitochondrial bioenergetics may be an important target for improving ischemia tolerance, and SS-20 may serve well for minimizing acute kidney injury and chronic kidney disease following surgical procedures such as partial nephrectomy and transplantation.

acute kidney injury; chronic kidney disease; ischemia-reperfusion injury; mitochondrial cristae; mitochondrial swelling; Szeto-Schiller peptides; SS-20

PARTIAL NEPHRECTOMY is recommended as standard treatment for small renal tumors as it carries a lower risk of chronic kidney disease compared with radical nephrectomy (9, 27). Warm ischemia time is thought to be a major factor in determining renal dysfunction after partial nephrectomy (5, 38, 53, 54). Extending warm ischemia time increases the risk of acute renal failure and chronic kidney disease, and an expert panel of the Society of Urologic Oncology concluded that every minute of warm ischemia has a deleterious effect on renal function outcomes following partial nephrectomy (38). Kidney cooling, which increases ischemia tolerance during open partial nephrectomy, is not as readily used during laparoscopic partial nephrectomy. Consequently, there is much interest in developing pharmacological approaches to improve ischemia tolerance (23). Ischemia leads to rapid depletion of ATP and dramatic loss of cellular ATP is noted within 5–10 min of renal ischemia (45). Decline in intracellular ATP inhibits ATP-dependent processes in the cell, including ion transport and cytoskeletal organization. In ischemia, Na\(^{+}\)-K\(^{+}\)-ATPase activity is inhibited within a few minutes and intracellular Na\(^{+}\) can increase three- to fourfold, resulting in cell swelling in the medullary thick ascending limb (mTAL) (2, 7, 33, 39). ATP is also required for actin polymerization and the drop in ATP results in rapid breakdown of the brush border, loss of cell polarity and cell-cell contact, and cell detachment in proximal tubular (PT) cells (3, 7). The redistribution of the Na\(^{+}\)-K\(^{+}\)-ATPase from the basal membrane inhibits Na\(^{+}\) reabsorption (35), and we previously reported that fractional excretion of Na\(^{+}\) is dramatically elevated after 45-min ischemia but not affected by 30-min ischemia (51). Cell death is usually restricted to the outer medullary region where oxygen tension drops precipitously at the corticomedullary junction (8, 14). In the rat, 30-min ischemia caused apoptosis and focal necrosis in the outer stripe of the outer medulla (OSOM), whereas 45-min ischemia resulted in extensive tubular necrosis and cell sloughing (51). Thus, prolonging warm ischemia time from 30 to 45 min significantly worsens acute kidney injury in the rat.

During ischemia, cell swelling caused by ATP depletion increases the osmotic gradient that drives water into the mitochondrial matrix to cause matrix swelling (21). Thirty minutes of renal ischemia in rats increased matrix volume threefold and caused unfolding of cristae membranes (7). The loss of cristae membranes inhibits mitochondrial respiration and slows down the recovery of ATP synthesis upon reperfusion (31, 51). Furthermore, electron leak in the mitochondrial ETC is a major source of reactive oxygen species (ROS) after ischemia (10, 32). Increased mitochondrial ROS can cause cardiolipin peroxidation and promote mitochondrial permeability transition, resulting in loss of cytochrome c and inhibition of mitochondrial respiration (15).

Although much attention has focused on the use of antioxidants and inhibitors of mitochondrial permeability transition as therapies to minimize ischemia-reperfusion injury, there have been limited attempts to promote ATP synthesis during and after ischemia. We recently reported on a mitochondria-targeted tetrapeptide (SS-31) that can accelerate ATP recovery after renal ischemia and significantly improves renal function

Address for reprint requests and other correspondence: H. H. Szeto, Dept. of Pharmacology, Weill Cornell Medical College, 1300 York Ave., New York, NY 10021 (e-mail: hh.szeto@med.cornell.edu).

http://www.ajprenal.org
1931-857X/15 Copyright © 2015 the American Physiological Society
F11
(7, 51). However, SS-31 can also scavenge ROS and inhibit mitochondrial permeability transition, and these actions were thought to be the mechanisms behind the reduction in ischemia-reperfusion injury (12, 13, 29, 59). In this paper, we show that another mitochondria-targeted peptide, SS-20, which does not have ROS scavenging capacity and does not inhibit mitochondrial permeability transition (59), can protect mitochondrial structure during ischemia and improve coupling efficiency of mitochondrial respiration even after 45-min ischemia. The ability of SS-20 to boost ATP synthesis during ischemia enhances ischemia tolerance and minimizes both acute renal dysfunction and chronic interstitial fibrosis. MATERIALS AND METHODS

Chemicals and materials. SS-20 (H-Phe-D-Arg-Phe-Lys-NH₂) was provided by Stealth Peptides (Newton Centre, MA). Unless specified, all other reagents were obtained from Sigma (St. Louis, MO).

Rat model of renal ischemia-reperfusion injury. All protocols had received prior approval by the Cornell University Institutional Animal Care and Use Committee. Care of the rats before and during the experimental procedures was conducted in accordance with the policies of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Adult male Sprague-Dawley rats (250 to 300 g) were anesthetized with 90 mg/kg ketamine and 4 mg/kg xylazine. Bilateral renal ischemia was induced by the application of nontraumatic microvascular clamps around both left and right renal pedicles for 30 or 45 min, as described in detail previously (7, 51). Sham-operated animals were not subjected to ischemia. Animals were randomly assigned to the following groups: sham-operated, ischemia-reperfusion with saline, or ischemia-reperfusion with SS-20. For acute treatment, SS-20 (2 mg/kg) or saline was administered subcutaneously 30 min before onset of ischemia and at the onset of reperfusion. SS-20 is a structural analog of SS-02 and SS-31, and these peptides have been shown to permeate all cell types, including renal epithelial cells (58). Systemic administration in rodents results in rapid tissue distribution, with significant uptake into all major organs within 30 min, and selectivity concentrating in the kidney (7). The dose of SS-20 was selected based on a previous report showing that SS-20 is as effective as SS-31 in preventing myocardial ischemia-reperfusion injury in the rat (11), as well as dose-response studies with SS-31 in this renal ischemia model (51). For chronic treatment, rats were exposed to SS-20 for 4 wk after ischemia with a subcutaneously implanted osmotic pump (Alzet model 2004, Cupertino, CA). Serum and urine samples were collected at 24 h after ischemia.

Animals were killed and kidneys were harvested at various times after reperfusion for biochemical and histological analysis. Kidney tissues were frozen at −80°C for Western blot or embedded in OCT cryostat sectioning medium containing 30% sucrose for immunohistochemistry. Immunohistochemical staining for E-cadherin and β₁-integrin was performed using anti-E-cadherin or anti-β₁-integrin antibodies (Abcam, Cambridge, MA) (7). For E-cadherin, we used the following scoring system: 1 (sharply delineated staining between cells), 2 (clear staining between cells but with some diffusion), 3 (some staining between cells and weak cytoplasmic staining), 4 (cytoplasmic distribution with weak staining between cells), and 5 (diffuse cytoplasmic distribution without clear staining between cells). For β₁-integrin staining, the following scoring system was used: 1 (sharply delineated staining on basal surface), 2 (clear basal staining with some diffusion to lateral surface), 3 (weak cytoplasmic staining with some loss of basal staining), 4 (cytoplasmic distribution with weak basal staining), and 5 (diffuse cytoplasmic distribution with staining on apical surface).

Other samples were fixed in 4% paraformaldehyde for paraffin sections. Sections from the outer medulla were assessed using PAS staining. For scoring cell swelling in the inner stripe of the outer medulla (ISOM), five fields (×200) were obtained from each of four animals for each condition (sham, saline, and SS-20). The following scoring system was used to quantify percent tubules with swollen cells: 1 (<20%), 2 (21–40%), 3 (41–60%), 4 (61–80%), or 5 (>81%). The score was averaged for each animal and subjected to statistical analysis. Masson's trichrome stain was used to assess interstitial fibrosis, and the percent of area staining blue was quantified by Image J (NIH) for 10 different fields and averaged (33).

Kidneys were harvested at the end of ischemia for ultrastructural examination by transmission electron microscopy (JEOL JEM-1400) (3 animals in each group). For each sample, representative epithelial cells from three mTAL and three PT segments were examined for mitochondrial morphology. Five representative mitochondria obtained at ×80,000 magnification were selected from each cell, and efforts were made to quantify mitochondrial size and morphology. Image J software was used to determine mitochondrial area, mitochondrial density, matrix density, and cristae morphology. The electron microscopic images were converted to binary files and mean gray score was used to determine mitochondrial density. For matrix density, five representative matrix areas were selected and their density was averaged for the individual mitochondrion. Cristae pathology was scored by two investigators based on percentage of collapsed or swollen cristae: 1, <20%; 2, 20–40%; 3, 40–60%; 4, 60–80%; 5, >80%. The mitochondrial measurements from the five mitochondria were averaged for each cell.

Mitochondrial respiration studies. Kidney mitochondria were isolated from whole kidneys after 45-min ischemia and O₂ consumption was measured using a Clark electrode (Hensatech, Norfolk, UK) as described previously (6, 7). Freshly isolated mitochondria (40 μg protein) were incubated with 400 μM ADP at 37°C for 1 min (representing state 2 respiration), after which glutamate/malate (0.5 mM) or succinate (0.5 mM) was added to initiate state 3 respiration. Mitochondrial respiration was allowed to proceed to state 4, when both endogenous and exogenous ADP has been converted to ATP. The respiratory control ratio (RCR) was calculated as a ratio of state 3 over state 4 respiration. Whole kidneys were homogenized and tissue ATP content was determined using the ATP Bioluminescent Assay Kit (Sigma).

Statistical analysis. Results are expressed as means ± SE. Statistical analysis was carried out using Prism software (GraphPad Software, San Diego, CA). Multiple group comparisons were performed using ANOVA followed by Tukey's post hoc test. Student's t-test was used for comparison between two groups. A P < 0.05 was considered statistically significant.

RESULTS

SS-20 protects tubular structure during ischemia. The mTAL segments in the ISOM can readily tolerate 30 min of ischemia with no significant damage to cellular structure or tubularchitectonics (Fig. 1, top). Increasing ischemia time to 45 min caused extensive cell swelling in the mTAL. PT cells in the OSOM showed disrupted cytoarchitecture but little cell swelling after 45-min ischemia (Fig. 1, bottom). The most prominent change in PT cells is the thinning of the brush border and fragments of shed brush border could be seen in the lumen.

Electron microscopy showed dramatic cell swelling in the mTAL after 45-min ischemia, and this was accompanied by rounded and swollen mitochondria (Fig. 2A). They also appear disorganized because of the loss of basal membrane invaginations. In contrast to the saline-treated animals, tubular architecture was well-preserved after 45-min ischemia in the SS-20-treated animals, and there was only some loss of cytoplasmic density in the mTAL while mitochondria remained

AJP-Renal Physiol • doi:10.1152/ajprenal.00366.2014 • www.ajprenal.org
elongated with normal density (Fig. 2B). Electron microscopy confirmed the lack of cell swelling in the PT, but brush borders are very short and rounded mitochondria can be seen throughout the cell (Fig. 2C). In the SS-20-treated animals, brush borders are preserved and mitochondria appear to have more density (Fig. 2D).

**SS-20 protects mitochondrial structure during ischemia.** Mitochondria underwent dramatic morphological changes after 45-min ischemia in both mTAL and PT segments. Representative electron microscopic images obtained at ×80,000 magnification are shown in Fig. 3. In addition to being large and rounded, the most commonly observed changes were cristae swelling (Fig. 3F) and matrix swelling with loss of cristae membranes (Fig. 3B, C, G). With severe matrix swelling, some of the inner mitochondrial membrane (IMM) becomes so stretched that it is compressed against the outer mitochondrial membrane (OMM). This results in the loss of most of the cristae membranes and the remaining cristae are very short and condensed. This is particularly dramatic in the mTAL. The OMM is also stretched and some visible breaks can be observed in the OMM of a few mitochondria, but in the majority of mitochondria the OMM remains intact. Pretreatment with SS-20 before onset of ischemia protected mitochondrial structure in both mTAL and PT segments. In most mTAL segments, mitochondria remained elongated with many cristae membranes (Fig. 3D). Although most mitochondria were still rounded in the PT, they were filled with cristae membranes and had more condensed matrix density (Fig. 3H).

The effects of SS-20 treatment on mitochondrial swelling and cristae architecture are summarized in Fig. 4. Three saline-treated and three SS-20-treated rats were included in the analysis. For each animal, three mTAL and three PT epithelial cells were selected for examination. Mitochondrial area, cristae density, and matrix density were averaged from five representative mitochondria in these cells. Cristae pathology was scored based on incidence of cristae swelling or cristae collapse. SS-20 treatment significantly reduced mitochondrial swelling, as shown by the reduction in mitochondrial area and increase in matrix density. SS-20 also increased cristae density and preserved normal cristae architecture.

**SS-20 improves mitochondrial function after ischemia.** Mitochondrial matrix swelling and loss of cristae membranes significantly inhibited mitochondrial function after ischemia. Mitochondria isolated from saline-treated ischemic kidneys...
showed significant reduction in state 3 respiration and RCR using either complex I (glutamate/malate) or complex II (succinate) substrates (Fig. 5A). This inhibition of mitochondrial function greatly delayed the recovery of ATP synthesis upon reperfusion and tissue ATP levels remained significantly depressed even after 1-h reperfusion (Fig. 5B). By protecting mitochondrial structure during ischemia, SS-20 treatment resulted in significant improvement of both state 3 respiration and RCR (Fig. 5A), and more rapid restoration of ATP levels upon reperfusion (Fig. 5B).

SS-20 promotes rapid recovery of ATP-dependent cellular processes in tubular cells. ATP is required for maintaining the actin cytoskeleton that is responsible for cell-cell contact and cell attachment in PT (44). Ischemia of 45-min duration led to cytosolic redistribution of E-cadherin from the lateral membrane in the saline-treated animals (Fig. 6A). With SS-20 pretreatment, some E-cadherin staining could still be found between tubular cells at the end of ischemia. Within 5 min of reperfusion, E-cadherin can be clearly seen between tubular cells in SS-20-treated animals while it appears on the apical surface in saline-treated animals. Figure 6B shows SS-20 significantly preserved the localization of β1-integrin to the lateral surface of the PT cells.

The delay in ATP recovery after ischemia is also seen in the mTAL, where cell swelling persisted in the ISOM even 1 h after onset of reperfusion (Fig. 7A). In contrast, cell swelling was significantly reduced in the rats treated with SS-20 (Fig. 7B).

Effect of SS-20 pretreatment on acute ischemic kidney injury. This model of bilateral warm ischemia results in significant tubular cell injury and renal dysfunction in rats, and the extent of injury is directly correlated with ischemia time (51). Thirty-minute ischemia did not alter fractional excretion of Na⁺ (FENa) but 45-min ischemia increased FENa more than 12-fold the day after ischemic injury. In this study, similar changes in renal biomarkers were observed in the saline-treated animals with 45-min ischemia (Table 1). Pretreatment with SS-20 30 min before ischemia significantly attenuated all changes to levels we previously reported for 30-min ischemia (51), suggesting that SS-20 increased ischemia tolerance. Renal function was not determined at 4 wk after ischemia because we previously found complete normalization of renal biomarkers by 4 wk (33).
SS-20 treatment prevents tissue remodeling after acute ischemia. Despite recovery of renal function within 1 wk after acute ischemic injury, the kidney is known to undergo progressive fibrotic changes (33). Renal interstitial fibrosis, as indicated by Masson trichrome staining, was significantly increased in saline-treated kidneys 4 wk after ischemic insult compared with sham, and this was significantly attenuated by SS-20 treatment (Fig. 8).

Fig. 3. SS-20 protects mitochondrial structure during ischemia. Rats were subcutaneously treated with saline or SS-20 (2 mg/kg) 30 min before occlusion of renal blood flow bilaterally for 45 min. Kidney sections obtained after ischemia were examined by transmission electron microscopy (×80,000). Mitochondria from mTAL of sham animals are elongated with densely packed cristae membranes (A). Mitochondria from saline-treated rats after 45-min ischemia are grossly swollen, and cristae are either swollen (B) or short and collapsed (C). Mitochondria from mTAL of SS-20-treated rats remain elongated with numerous cristae membranes (D). Mitochondria from PT of sham animals are also elongated, although cristae are not as densely packed as in mTAL (E). Cristae swelling is observed in ~30% of PT mitochondria after 45-min ischemia (F), while others show matrix swelling and loss of cristae membranes (G). Although most PT mitochondria are rounded after SS-20 treatment, there is much less matrix swelling and cristae membranes are preserved (H).

Fig. 4. SS-20 protects mitochondrial morphology during ischemia. Rats were subcutaneously treated with saline or SS-20 (2 mg/kg) 30 min before occlusion of renal blood flow bilaterally for 45 min. Kidney sections were obtained after 45-min ischemia and subjected to electron microscopic examination at ×80,000 magnification. Top: Pretreatment with SS-20 before ischemia significantly reduced mitochondrial swelling as indicated by decrease in mitochondrial area and increase in matrix density. Pretreatment with SS-20 also significantly preserved cristae structure as shown by increase in mitochondrial density and decrease in cristae pathology. *P < 0.05. **P < 0.01. ***P < 0.001.
Continuous ATP production is necessary for the kidney to carry out its crucial task of fluid regulation and waste excretion. The duration of ischemia that can be tolerated by the kidney depends on the amount of ATP that can be produced with residual substrates and oxygen in the tissue after cessation of blood flow. In the kidney, complete ischemia has been reported to reduce ATP levels 70–90% in 10 min (45). We previously reported that the rat kidney tolerates 30-min ischemia quite well with injury limited to apoptosis and focal necrosis in the OSOM (51). In contrast, extending ischemia time to 45 min resulted in extensive necrosis and cell sloughing in the OSOM, and tubules in the ISOM were clogged with cell debris and hyaline casts (51). In this study, tubular damage following 45-min ischemia was reflected in significant renal dysfunction 24 h later. However, pretreatment with a single dose of SS-20 30 min before ischemia prevented renal injury from 45-min ischemia, and thus SS-20 is able to extend ischemia tolerance from 30 to 45 min.

Tubular injury in acute ischemia has mainly been found in two segments in the outer renal medulla—the PT and mTAL. Proximal tubules are vulnerable to ischemia because they have minimal glycolytic capacity and must rely on mitochondrial metabolism for ATP synthesis (55). The mTAL segment is vulnerable to ischemia because of its very low blood flow and O2 supply. In addition to low blood flow, this part of the nephron also has high ATP demand for Na\(^{+}\)/H\(^{+}\) transport (8, 14).

It is significant that fractional excretion of Na\(^{+}\)/H\(^{+}\) and K\(^{+}\)/H\(^{+}\) were unchanged 1 day after the kidneys were subjected to 30-min ischemia (51), confirming that injury to the medullary tubules is limited and full function of the Na\(^{+}\)/H\(^{+}\)-K\(^{+}\)/H\(^{+}\)-ATPase can be restored upon reperfusion. There was dramatic increase in fractional excretion of Na\(^{+}\) and serum K\(^{+}\) after 45-min ischemia, indicating dysfunctional Na\(^{+}\)-K\(^{+}\)-ATPase. Here, we show that pretreatment with SS-20 is capable of protecting the function of the Na\(^{+}\)-K\(^{+}\)-ATPase even with 45-min ischemia and fractional excretion of Na\(^{+}\) and K\(^{+}\) is comparable with those we reported for 30-min ischemia (51).

The Na\(^{+}\)-K\(^{+}\)-ATPase plays a crucial role in electrolyte transport and fluid regulation in the kidney. The kidney is rich in Na\(^{+}\)-K\(^{+}\)-ATPase, especially the mTALs, and they account for 80% of O2 consumption in the kidney and 50% of ATP...
expenditure (14, 24, 26, 30, 34). ATP depletion during ischemia would therefore greatly impact on the Na\(^+\)/H\(^+\)-ATPase and intracellular Na\(^+\) can increase three- to fourfold in a few minutes and cause cell swelling (28). Dramatic cell swelling was observed in the mTAL after 45-min warm ischemia but not after 30-min ischemia. Interestingly, no mTAL cell swelling was observed with 45-min ischemia when animals were pretreated with SS-20, suggesting that there must be sufficient cellular ATP for the Na\(^+\)/K\(^+\)-ATPase to regulate intracellular Na\(^+\) in the mTAL. Improved preservation of cellular ATP is also evident in the PT. In the PT, breakdown of the cytoskeleton is more pronounced than cell swelling (7, 51). Pretreatment with SS-20 preserved brush border and helped maintain cell-cell contact via E-cadherin and cell attachment to the basement membrane via β\(_1\)-integrin.

In addition to cell swelling, our results show that mitochondria undergo dramatic matrix swelling during ischemia. There is a direct relationship between mitochondrial matrix volume and osmolarity of the cytoplasm (42). This matrix swelling most likely results from osmotic balance between the swollen cytoplasmic compartment and mitochondrial matrix, as both the OMM and IMM are freely permeable to water, which can also follow Na\(^+\) entry into the matrix through the Na\(^+\)/H\(^+\) exchanger (19, 20). Matrix volume can increase until the IMM becomes completely unfolded into a spherical configuration and the OMM ruptures because the area of the IMM is at least...
four times larger than the OMM. Varying degrees of matrix swelling and unfolding of the IMM were observed in mTAL in saline-treated kidneys. In many instances, the IMM was stretched and compressed against the OMM, and only very short condensed cristae or swollen cristae were apparent, indicating inactive state of mitochondrial respiration. The more pronounced mitochondrial matrix swelling in mTAL compared with PT may be related to the energy dependence of sodium reabsorption in the mTAL.

Pretreatment with SS-20 before ischemia prevented cell swelling and mitochondrial swelling in mTAL, and cristae morphology was preserved even after 45-min ischemia. These findings support the idea that mitochondrial matrix swelling is secondary to cell swelling caused by inhibition of Na⁺-K⁺-ATPase, and they suggest that SS-20 is able to provide sufficient intracellular ATP to tolerate 45-min ischemia. It should be pointed out that mTAL segments do have capacity for anaerobic glycolysis, and this likely helps to sustain some cytosolic ATP production to maintain osmotic regulation during ischemia. Upregulation of glycolytic enzymes by stabilizing hypoxic-inducible transcription factors has been shown to increase glycolysis and preserve mTAL function following ischemia-reperfusion injury (43). The effects of SS-20 on anaerobic glycolysis are not known, but it is unlikely that administration of SS-20 30 min before onset of ischemia would change enzyme levels or activity. In contrast, PT have little capacity for glycolysis and are dependent on oxidative phosphorylation for ATP supply (55). Furthermore, mitochondria in the PT are more susceptible to O₂ depletion than mTAL (16, 17). Thus, SS-20 is more likely to increase mitochondrial

Fig. 7. SS-20 prevents medullary tubular cell swelling after ischemia-reperfusion. Rats were treated with saline or SS-20 (2 mg/kg) 30 min before occlusion of renal blood flow bilaterally for 45 min. Treatment was repeated just before onset of reperfusion. Kidney sections were obtained after 60-min reperfusion and stained with PAS. A: representative sections from the ISOM are shown from a sham-operated kidney, saline-treated ischemic kidney, and SS-20-treated ischemic kidney (top, ×200). Swollen tubular cells are prevalent in the saline-treated samples and greatly reduced in the SS-20-treated samples. The boxed areas are magnified in the bottom panels. B: percent of tubules with swollen cells was quantified as described in MATERIALS AND METHODS. The scores from 5 different fields were averaged for each animal with 4 animals in each group. ***P < 0.001.
activity to improve ATP production for cell survival under ischemic conditions.

The mechanism by which SS-20 increases mitochondrial ATP production during ischemia is not completely understood. SS-20 belongs to the family of Szeto-Schiller (SS) peptides, synthetic tetrapeptides with an alternating aromatic-cationic motif (52, 59). These are cell-permeable peptides that selectively target mitochondria and concentrate in the IMM (30, 57, 59). We recently discovered that these peptides bind specifically to cardiolipin, a unique phospholipid that is exclusively expressed on the IMM. Cardiolipin is required for proper cristae formation and for stabilization of respiratory supercomplexes for more efficient electron transfer (25, 36, 40, 56).

Cardiolipin also serves to anchor cytochrome c to the IMM via electrostatic interaction to facilitate electron transport from complex III to complex IV. However, ~20% of cytochrome c interacts hydrophobically with cardiolipin. This [cytochrome c/cardioli- pin] complex results in unfolding of cytochrome c and disrupts the Met80-heme iron coordination, thereby converting cytochrome c from an electron carrier to a peroxidase that cannot participate in electron transfer (4, 18, 22, 41, 47). This hydrophobic interaction between cytochrome c and cardiolipin is further enhanced by low ATP concentrations (46, 48) and may contribute to inhibition of the electron transport chain during ischemia. The peroxidation of cardiolipin would further reduce electrostatic interaction with cytochrome c and inhibit the electron transport chain. A recent study showed that ROS generated by mitochondria during ischemia oxidizes Met80 and promotes cytochrome c peroxidase activity, resulting in a defective electron transport chain (1). We reported that SS-31, another member of SS peptide family, can stabilize the Met80-heme iron coordination and improve electron transfer through this [cytochrome c/cardiolipin] complex (6). Thus, SS-20 may also facilitate electron transfer through this [cytochrome c/cardiolipin] complex and accelerate electron transfer from complex III to complex IV during ischemia. This would effectively increase mitochondrial ATP production, and reduce electron leak and superoxide generation, under ischemic conditions. This extra boost of ATP production may be sufficient to increase ischemia tolerance from 30 to 45 min.

The loss of cristae during ischemia, together with dilution of substrates due to matrix swelling, serves to inhibit mitochondrial respiration upon reperfusion. Indeed, studies with isolated mitochondria obtained at the end of ischemia showed significantly reduced state 3 respiration and uncoupling of oxidative phosphorylation even in the presence of adequate oxygen and substrates. Tissue ATP levels remain very low even after 1-h reperfusion in saline-treated animals. Both E-cadherin and β1-integrin failed to redistribute to their normal positions during early reperfusion, and mTAL cells remained swollen even after 60 min of reperfusion.

By preventing mitochondrial swelling, the SS-20-treated ischemic mitochondria had significantly better state 3 respiration and coupling between oxygen consumption and ATP production that cannot participate in electron transfer (4, 18, 22, 41, 47). This hydrophobic interaction between cytochrome c and cardiolipin is further enhanced by low ATP concentrations (46, 48) and may contribute to inhibition of the electron transport chain during ischemia. The peroxidation of cardiolipin would further reduce electrostatic interaction with cytochrome c and inhibit the electron transport chain. A recent study showed that ROS generated by mitochondria during ischemia oxidizes Met80 and promotes cytochrome c peroxidase activity, resulting in a defective electron transport chain (1). We reported that SS-31, another member of SS peptide family, can stabilize the Met80-heme iron coordination and improve electron transfer through this [cytochrome c/cardiolipin] complex (6). Thus, SS-20 may also facilitate electron transfer through this [cytochrome c/cardiolipin] complex and accelerate electron transfer from complex III to complex IV during ischemia. This would effectively increase mitochondrial ATP production, and reduce electron leak and superoxide generation, under ischemic conditions. This extra boost of ATP production may be sufficient to increase ischemia tolerance from 30 to 45 min.

Table 1. Renal function 24 h after ischemia-reperfusion

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Sham</th>
<th>Saline</th>
<th>SS-20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum creatinine, mg/dl</td>
<td>0.39 ± 0.02</td>
<td>3.05 ± 0.16*</td>
<td>1.57 ± 0.24**</td>
</tr>
<tr>
<td>BUN, mg/dl</td>
<td>13.25 ± 0.59</td>
<td>105.9 ± 4.82*</td>
<td>64.00 ± 6.42**</td>
</tr>
<tr>
<td>FEna, %</td>
<td>0.20 ± 0.03</td>
<td>11.75 ± 2.00*</td>
<td>1.07 ± 0.16†</td>
</tr>
<tr>
<td>FEk, %</td>
<td>21.7 ± 2.0</td>
<td>218.8 ± 18.7*</td>
<td>95.3 ± 17.0f</td>
</tr>
<tr>
<td>Serum K+, meq/l</td>
<td>4.61 ± 0.09</td>
<td>6.62 ± 0.39*</td>
<td>4.62 ± 0.06†</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE (n = 6). *P < 0.001 compared with sham-operated control. †P < 0.001 compared with saline-treated ischemia.

Representative trichrome-stained sections (×100) from the OSOM are shown from sham (A), saline-treated (B), and SS-20-treated (C) animals. C, right: SS-20 treatment significantly reduced trichrome stain 4 wk after ischemia. ***P < 0.001, n = 6.
production. This allowed rapid redistribution of E-cadherin and β1-integrin to their proper locations within 5 min of reperfusion, thereby protecting epithelial cell-cell contact and attachment to the basement membrane. ATP content after 1 h of reperfusion was significantly higher in the SS-20-treated kidneys, although it was still lower than in sham kidneys due to increased ATP demand to repair cellular damages caused by ischemic injury. Importantly, the SS-20-treated animals showed only minor renal dysfunction 24 h after ischemia. Recovery from acute kidney injury is often not complete and progresses to chronic kidney disease with interstitial fibrosis. We showed that ischemic injury to the renal microvasculature results in chronic tissue hypoxia and persistent inflammation (33). Our results show that SS-20 not only significantly reduced acute renal dysfunction after prolonged ischemia, it is also very effective in preventing chronic interstitial fibrosis. These aromatic-cationic peptides distribute rapidly to all major organs after systemic administration, and they achieve highest levels in the kidney (49, 52). It should be emphasized that ischemia duration in a rodent model cannot be directly extrapolated to the human. The human kidney may tolerate ischemia better than rats, but very similar mitochondrial injuries with prominent swelling were seen in human biopsied kidney slices obtained after ~30-min ischemia (37). Thus, the use of SS-20 may serve well for minimizing acute kidney injury and chronic kidney disease following surgical procedures such as partial nephrectomy and transplantation.

Current efforts in therapeutic discovery for ischemia-reperfusion injury have mostly focused on minimizing oxidative stress and preventing mitochondrial permeability transition rather than on promoting bioenergetics. SS-20 lacks electron scavenging ability and does not inhibit calcium-induced mitochondrial permeability transition (27, 53, 57), and yet our study shows that SS-20 is very effective in minimizing ischemic injury in the kidney and it does so by preserving mitochondrial structure under ischemic conditions. SS-20 has also been reported to minimize ischemic injury in the heart, although the mechanism of action was not known at that time (11, 50). Careful examination of postischemic mitochondria in this study revealed loss of mitochondrial cristae rather than mitochondrial permeability transition as the primary cause of ischemia-reperfusion injury. Thus, bioenergetic failure may be more important than oxidative stress and the opening of the mitochondrial permeability transition pore in determining recovery from ischemic injury. These new findings reveal that enhancing mitochondrial bioenergetics may be an important target for improving ischemia tolerance.

ACKNOWLEDGMENTS

We thank Lee Cohen-Gould and her staff at the Electron Microscopy and Histology Core Facility at Weill Cornell Medical College. We especially thank Surya V. Seshan for advice on renal pathology.

GRANTS

This research was supported by the Research Program in Mitochondrial Therapeutics at Weill Cornell Medical College.

DISCLOSURES

The SS peptides described in this article are licensed for commercial research and development to Stealth Peptides Inc, a clinical stage biopharmaceutical company, in which HHS, AVB and the Cornell Research Foundation have financial interests. The Research Program in Mitochondrial Therapeutics was established with a gift from Stealth Peptides Inc.

AUTHOR CONTRIBUTIONS

Author contributions: H.H.S. conception and design of research; H.H.S., S.L., Y.S., and A.V.B. analyzed data; H.H.S., S.L., and A.V.B. interpreted results of experiments; H.H.S., S.L., and A.V.B. prepared figures; H.H.S., S.L., and A.V.B. drafted manuscript; H.H.S., S.L., Y.S., and A.V.B. edited and revised manuscript; H.H.S., S.L., Y.S., and A.V.B. approved final version of manuscript; S.L., Y.S., and A.V.B. performed experiments.

REFERENCES


