NOVEL CARDIOLIPIN THERAPEUTIC PROTECTS ENDOTHELIAL MITOCHONDRIA
DURING RENAL ISCHEMIA AND MITIGATES MICROVASCULAR RAREFACTION,
INFLAMMATION AND FIBROSIS

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Running head: SS-31 minimizes microvascular rarefaction and fibrosis

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Abstract

Microvascular rarefaction, or loss of microvascular density, is increasingly implicated in the progression from acute ischemic kidney injury to chronic kidney disease. Microvascular dropout results in chronic tissue hypoxia, interstitial inflammation and fibrosis. There is currently no therapeutic intervention for microvascular rarefaction. We hypothesize that capillary dropout begins with ischemic damage to endothelial mitochondria due to cardiolipin peroxidation, resulting in loss of cristae and the failure to regenerate ATP upon reperfusion. SS-31 is a cell-permeable peptide that targets the inner mitochondrial membrane and binds selectively to cardiolipin. It was recently shown to inhibit cardiolipin peroxidation by cytochrome c peroxidase activity, and it has been shown to protect mitochondrial cristae in proximal tubular cells during ischemia, and accelerated ATP recovery upon reperfusion. We found mitochondrial swelling and loss of cristae membranes in endothelial and medullary tubular epithelial cells after 45 minutes ischemia in the rat. The loss of cristae membranes limited the ability of these cells to regenerate ATP upon reperfusion and led to loss of vascular integrity and tubular cell swelling. SS-31 prevented mitochondria swelling and protected cristae membranes in both endothelial and epithelial cells. By minimizing endothelial and epithelial cell injury, SS-31 prevented “no-reflow” after ischemia and significantly reduced the loss of peritubular capillaries and cortical arterioles, interstitial inflammation, and fibrosis at four weeks after ischemia. These results suggest that mitochondria protection represents an upstream target for pharmacological intervention in microvascular rarefaction and fibrosis.
**Keywords:** acute kidney injury, chronic kidney disease, mitochondrial cristae, Szeto-Schiller peptides, SS-31.

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AKI</td>
<td>acute kidney injury</td>
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<tr>
<td>α-SMA</td>
<td>α smooth muscle actin</td>
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<tr>
<td>BUN</td>
<td>blood urea nitrogen</td>
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<td>CKD</td>
<td>chronic kidney disease</td>
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<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
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<tr>
<td>IR</td>
<td>ischemia-reperfusion</td>
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<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
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<tr>
<td>mTAL</td>
<td>medullary thick ascending limb</td>
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<td>TNFα</td>
<td>tumor necrosis factor α</td>
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<tr>
<td>TGFβ</td>
<td>transforming growth factor β</td>
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<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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Introduction

Acute ischemic kidney injury is becoming increasingly common in critically-ill patients and is associated with increased morbidity and mortality (45). Recovery from acute kidney injury (AKI) is often not complete and is marked by residual structural damage, including glomerulosclerosis, tubular atrophy, peritubular capillary rarefaction, and interstitial fibrosis. A recent systematic review and meta-analysis concluded that patients who survive AKI have a greater risk for chronic kidney disease (CKD), end-stage renal disease, and other adverse outcomes (10). Furthermore, the study revealed that the relationship between AKI and CKD was graded, with a greater risk associated with increasing severity of AKI.

Therapeutic interventions that minimize ischemia-reperfusion (IR) injury may therefore reduce the severity of CKD. Unexpectedly, a number of interventions that minimize acute IR injury either had no effect or actually worsened the progression to CKD. Inhibition of p53 with pifithrin-α diminished tubular cell apoptosis and protected renal function acutely, but provided no protection against microvascular rarefaction at 4 weeks and actually increased fibrosis at 8 weeks after ischemia (11). While hypoxia-inducing factor (HIF-1α) has been shown to be cytoprotective in AKI, prolonged activation of HIF-1α actually promoted fibrosis (22). Furthermore, chronic administration of the antioxidant tempol accelerated renal microvascular rarefaction and increased fibrosis in a model of renal artery stenosis (26).

Ischemic injury to the microcirculation is increasingly being recognized as the major factor in the pathogenesis of CKD (21, 25, 34, 35, 40). Experimental studies in rats and mice have revealed 30-50% loss of peritubular capillaries after acute ischemic injury (2, 23).
Reduced microvascular density has also been documented after kidney transplantation in humans (24, 44). The loss of capillary perfusion results in chronic tissue hypoxia causing further capillary and tubular damage, inflammation, and fibrosis (12, 17, 46).

We hypothesize that capillary dropout begins with ischemic damage to endothelial mitochondria and the failure to regenerate ATP upon reperfusion. Loss of ATP results in separation of endothelial tight junctions, detachment of endothelial cells from the basement membrane, endothelial blebbing, and endothelial necrosis (27). This causes increased capillary permeability, interstitial edema and inflammation. Furthermore, endothelial cell swelling, and leukocyte and platelet adhesion, result in capillary narrowing and reduced capillary flow. This leads to chronic tissue ischemia which aggravates endothelial degeneration and eventually leads to loss of the microvasculature (24, 50).

Thus protecting endothelial cells from the initial consequences of ATP depletion may be most effective in minimizing the downstream cascade of capillary injury.

We previously reported on a cardiolipin-targeted tetrapeptide (SS-31, also named Bendavia™) that protects tubular cell mitochondrial structure during ischemia and accelerates ATP recovery in a rat model of renal IR injury (6, 48). Here we report that SS-31 also protects endothelial cell mitochondria, reduces endothelial injury, minimizes "no-reflow", and significantly reduces microvascular rarefaction. As a result, there is a significant reduction in interstitial inflammation and fibrosis 4 weeks after acute IR injury. SS-31 represents a unique single approach to the multifactorial problem of "no-reflow", microvascular rarefaction, and fibrosis.
MATERIALS AND METHODS

Chemicals and Materials

SS-31 (D-Arg-dimethylTyr-Lys-Phe-NH₂) was supplied by Stealth Peptides Inc., Newton Centre, MA. Unless otherwise specified, reagents and assay kits were purchased from Sigma (St. Louis, MO).

Rat model of Renal IR Injury

Renal IR injury was induced as previously described (48). All protocols had received prior approval by the Cornell University Institutional Animal Care and Use Committee. Care of the animals before and during the experimental procedures was conducted in accordance with the policies of the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Male Sprague-Dawley rats (250-300 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. Ischemia was confirmed by blanching of the kidneys. After 45 min of ischemia, the clamps were removed and reperfusion was confirmed visually. Animals were randomly assigned to the following groups: (1) sham-operated animals; (2) ischemia with acute saline exposure; (3) ischemia with acute SS-31 exposure; or (4) ischemia with chronic SS-31 exposure. For acute exposure, SS-31 (2 mg/kg) or saline was administered subcutaneously 30 min before onset of ischemia and at the onset of reperfusion. For chronic exposure, rats were exposed to SS-31 for 4 weeks with a subcutaneously-implanted osmotic pump (Alzet model 2004, Cupertino, CA). Serum samples were collected at 24 hrs
and at weekly intervals up to four weeks after ischemia. All samples were stored at -20°C until analysis. Kidneys were harvested at four weeks and samples were frozen at -80°C for Western Blot or embedded in OCT cryostat sectioning medium containing 30% sucrose for immunohistochemistry. Other samples were fixed in 4% paraformaldehyde for paraffin sections. In a subset of animals (n=2) in each group, kidneys were harvested at the end of ischemia or after 5 min reperfusion for ultrastructural examination by transmission electron microscopy.

Transmission Electron Microscopy

Pieces of renal cortical and medullary tissue were fixed in 4% paraformaldehyde, post-fixed in 1% osmium tetroxide, dehydrated in graded alcohols, and embedded in Epon. Ultrathin sections (200 to 400 Å) were cut on nickel grids, stained with uranyl acetate and lead citrate, and examined using a digital electron microscope (JEOL USA, JEM-1400).

Histologic Evaluation

Paraffin sections were sliced at 3 μm, stained with PAS and examined by light microscopy (Nikon Eclipse, TE2000-U). Masson’s trichrome staining was used to assess interstitial fibrosis. Trichrome-stained sections were observed at x100 magnification, and the percent of area staining blue was quantified by Image J (NIH) for 10 different fields and averaged.

Assessment of the Renal Microvasculature
Microvascular density was assessed by immunohistochemical staining of CD31 for peritubular capillaries and \( \alpha \)-smooth muscle actin (\( \alpha \)SMA) for cortical arterioles. In addition, tissue expression of endothelial nitric oxide synthase (eNOS) and vascular endothelial growth factor (VEGF) were quantified by western blot.

Assessment of Interstitial Inflammation

Interstitial inflammation was assessed by immunohistochemical (IHC) staining for macrophages (CD68) and lymphocytes (CD3) using paraffin-embedded kidney sections. The number of CD68\(^+\) cells or CD3\(^+\) cells in the outer medulla were counted from five different fields for each sample and averaged. The inflammatory cytokines, tumor-necrosis factor \( \alpha \) (TNF\( \alpha \)) and transforming growth factor \( \beta_1 \) (TGF-\( \beta_1 \)) were quantified by western blot.

Immunohistochemistry and Immunofluorescent Staining

Paraffin sections (4 \( \mu \)m) were blocked in blocking buffer for 30 min at room temperature and incubated with anti-\( \alpha \)SMA (Dako, Carpinteria, CA), anti-CD68 (Abcam, Cambridge, MA), anti-CD3 (Dako, Carpinteria, CA) overnight at 4\(^{\circ}\)C. Secondary antibodies were HRP-conjugated anti-mouse IgG (Dako, Carpinteria, CA) or HRP-conjugated anti-rabbit IgG (Vector, Burlingame, CA) for 30 min at room temperature. Slides were developed with the DAB substrate kit (Vector, Burlingame, CA) and covered with Cytoseal XYL mounting medium. Frozen OCT blocks were cut into 8 \( \mu \)m sections, fixed in acetone for 5 min at room temperature and incubated with anti-CD31 (BD, Franklin Lakes, NJ) overnight at 4\(^{\circ}\)C, and then treated with Alexa Fluor-488-conjugated anti-mouse IgG (Invitrogen,
Eugene, OR) for 30 min at room temperature, and covered with VECTASHIELD Mounting Medium with DAPI (Vector, Burlingame, CA).

**Western Blot for VEGF, eNOS, TNFα and TGFβ1**

Tissue homogenates were prepared according to lysis protocol for radioimmunoprecipitation assay buffer (Santa Cruz Biotechnology, Santa Cruz, CA). Kidney homogenates (35 μg) were suspended in loading buffer and subjected to a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The resolved proteins were transferred to an Immun-Blot PVDF membrane (Bio-Rad, Hercules, CA). After electroblotting, the membrane was incubated overnight with anti-TNFα or anti-TGFβ1 (Novus, Littleton, CO), anti-VEGF or anti-eNOS (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. Membranes were further incubated for 1 hour with HRP-conjugated polyclonal rabbit anti-goat IgG or monoclonal goat anti-mouse IgG (Pierce, Rockford, IL). Protein bands were detected with an enhanced chemiluminescence detection system (Cell Signaling, Danvers, MA) and autoradiography. Bands were evaluated for integrated density values on Image Lab™ Software (Bio-Rad, Hercules, CA).

**Statistical Analysis**

All results are expressed as means ± SEM. Statistical analyses were carried out using Prism software (GraphPad Software, Inc., San Diego, CA). Multiple group comparisons were performed using ANOVA followed by a Tukey *post hoc* test. A P value less than 0.05 was considered statistically significant.
RESULTS

Endothelial Cell Damage during Acute IR Injury

The model of bilateral renal ischemia for 45 min results in significant renal dysfunction 24 hours later as measured by serum creatinine, blood urea nitrogen, and fractional Na+ excretion (48). To examine endothelial cell injury during acute IR, kidneys were harvested at the end of 45 minute ischemia, or after one hour reperfusion. Electron microscopy revealed structural changes to capillary endothelial cells in the outer medulla after 45 min ischemia in the saline group. Endothelial cells appeared enlarged and their cytoplasm contained many vacuoles (Figure 1A) that were identified as swollen mitochondria with loss of cristae membranes when examined at 30,000x magnification (Figure 1B). Figure 1C shows swollen mitochondria in a capillary endothelial cell and a degenerating interstitial cell that also contained swollen mitochondria. Within 5 min of reperfusion, some endothelial cells show early signs of detachment from the basement membrane, while others show clear degenerative changes (Figure 1D). Endothelial cell detachment damaged capillary integrity and led to interstitial edema and extravasation of red blood cells into the interstitial space (Figure 1E). Aggregates of platelets were observed in many peritubular capillaries with some completely blocking the capillary lumen even after 5 min reperfusion, resulting in stasis of red blood cells and “no-reflow” in the kidney (Figure 1F).

SS-31 Protects Endothelial Cells during IR and Mitigates Capillary Damage

Endothelial cells from SS-31 treated animals did not show ischemic injury even after 45 min ischemia (Figure 2A). The endothelial cells remained attached to the basement
membrane with cytoplasmic processes extending along the capillary wall (Figure 2B). The mitochondrion identified in the cytoplasm was examined under 80,000x magnification and revealed intact inner and outer mitochondrial membranes and stacks of cristae membranes (Figure 2C). Figure 2D shows the cytoplasmic processes of the endothelial cell extending around the capillary wall, and the highlighted area revealed normal mitochondria when examined under 30,000x magnification (Figure 2E). Within 5 min of reperfusion, blood flow was restored and the endothelial cell remained attached to the basement membrane (Figure 2F). Platelet aggregation was not seen in the SS-31 treated samples.

**SS-31 minimizes medullary tubular cell swelling and red blood cell trapping**

The medullary thick ascending limb (mTAL) is particularly susceptible to ischemic injury because of the low oxygen tension in the outer medulla and the high metabolic demands required for solute reabsorption (16). Cells of the mTAL have been shown to undergo mitochondrial swelling with ischemia and subsequently progress to cell death by apoptosis or necrosis (7, 41). Using electron microscopy, we found dramatic cell swelling in mTAL segments following 45 min ischemia (Figure 3A). The mitochondria were rounded and empty, with few cristae membranes (Figure 3B). They also appear very disorganized because of the loss of basal membrane invaginations. In contrast, the medullary tubular cells in animals treated with SS-31 were not swollen (Figure 3C). The mitochondria were elongated and surrounded by basal membrane invaginations, and cristae membranes were well-preserved even after 45 min of complete ischemia (Figure 3D).

Tubular cell swelling was prevalent in the saline group throughout the inner stripe of the outer medulla even after 1 hour of reperfusion (Figure 4, top panel). Tubular cell
swelling led to compression of the peritubular capillaries with trapping of red blood cells and disruption of capillary blood flow despite reperfusion (Figure 4, bottom panel). Because tubular cell swelling was prevented in the SS-31 animals, the lack of red cell trapping resulted in improved capillary blood flow (Figure 4).

Renal Microvascular Rarefaction 4 weeks after Ischemic Injury

Renal ischemia can result in permanent damage to peritubular capillaries, with the outer medulla being the most affected (2, 23, 46). A 30-40% loss of microvascular density has been reported 4 weeks after ischemic insult (30). We examined the long-term effects of acute ischemia on peritubular capillaries by immunohistochemical staining for CD31, a biomarker for endothelial cells. Quantitative image analyses show that there was a dramatic reduction in CD31 staining 4 weeks after ischemia when compared to the sham group (Figure 5A). CD31 staining was improved almost 4-fold in the SS-31 treated kidneys. We also examined the glomerular capillaries because a previous study using intravital videomicroscopy showed that glomerular capillary blood flow is also reduced following ischemia (49). The dramatic loss of glomerular capillaries 4 weeks after ischemia was improved 10-fold by acute administration of SS-31 during IR injury (Figure 5B). Ischemia can also cause a loss of cortical arterioles (52). We examined the number of cortical arterioles by immunostaining for α-smooth muscle actin (α-SMA). Figure 5C shows that SS-31 significantly reduced the loss of cortical arterioles 4 weeks after IR injury. The loss of endothelial cells after acute ischemia is reflected in the significant decrease in eNOS expression in the saline group, and this was significantly improved by pretreatment with
SS-31 (Figure 5D). Continuous treatment with SS-31 after ischemia did not provide any further protection of endothelial cells.

Loss of microvasculature after acute ischemic injury results in chronic tissue hypoxia, and it is well-known that hypoxia will stimulate the release of proangiogenic factors such as vascular endothelial growth factor (VEGF) (9). At 4 weeks after ischemic insult, we found a >10-fold increase in VEGF expression in the saline-treated kidneys and this was significantly reduced by SS-31 treatment (Figure 5E). Continuous treatment with SS-31 for 4 weeks did not result in any further change in VEGF expression.

Recovery of Renal Function after Acute Ischemia

We examined serum creatinine and BUN at weekly intervals up to four weeks after IR injury (n=7 in each group). In the saline-treated group, two rats died within 48 hours after ischemia. All animals that received SS-31, even only during the immediate IR period, survived at least four weeks after ischemia. Renal function for the surviving animals are summarized in Table 1. Serum creatinine and BUN were within normal limits in both groups within one week after ischemia, and there was no significant difference between the two groups thereafter. Renal function was not determined in the pump group that received SS-31 continuously after ischemia as the saline-treated animals had normal renal function at 4 weeks.

Renal Histopathology 4 weeks after Ischemic Injury

Despite the recovery of renal function within one week after acute IR injury, histopathology revealed chronic changes in the kidneys of saline-treated animals.
Pronounced tubular dilation, tubular cell atrophy, and thickening of the glomerular and
tubular basement membrane were observed in the saline group (Figure 6). These
pathological changes were greatly reduced in the animals that received SS-31 only during
the immediate IR injury (Figure 6). No additional improvement was found when SS-31
treatment was continued throughout the 4 week period (results not shown).

*SS-31 reduces Interstitial Inflammation 4 weeks after Ischemia*

Inflammation plays an important role in the initiation and extension of AKI. Acute
ischemic injury is associated with interstitial inflammation, with infiltration of
macrophages and neutrophils (48). Tumor-necrosis factor-α (TNF-α) is the most potent
mediator of inflammation, and it is upregulated and released after ischemia (42). We found
a significant upregulation of TNF-α in kidney tissues even 4 weeks after ischemia (Figure
7A). This was accompanied by a sustained increase in CD68+ macrophages and CD3+
lymphocytes in the peritubular interstitium (Figure 7B and 7C). The increase in TNF-α and
inflammatory cells were all prevented by SS-31 treatment at the time of acute IR injury
(Figure 7 A-C). Continuous administration of SS-31 for 4 weeks showed no further benefit
(Figure 7A).

*Tubulointerstitial Fibrosis 4 weeks after Ischemia*

The tubulointerstitial influx of inflammatory cells contribute to fibrosis by releasing
profibrotic cytokines (29). TGFβ is considered the key mediator of renal fibrosis (19, 43),
and its expression was upregulated 2.5-fold 4 weeks after IR injury, and this was
completely abolished by treatment with SS-31 during the immediate IR period (Figure 8A).
Continuous administration of SS-31 for 4 weeks showed no further benefit (Figure 8A). Renal fibrosis, as indicated by Masson trichrome staining, was increased significantly 4 weeks after acute ischemia when compared to sham (Figure 8B). Fibrosis was seen mostly in the outer medulla. The increase in trichrome staining was completely prevented by treatment with SS-31 only during the immediate IR injury, and continuous treatment with SS-31 for 4 weeks provided no further protection (Figure 8B). Myelofibroblasts are the dominant collagen-producing cells, and there was an increase in interstitial cells expressing α-smooth muscle actin (αSMA) 4 weeks after acute ischemia when compared to sham, and this was abolished by SS-31 treatment during the immediate IR period (Figure 8C).

**DISCUSSION**

Although direct injury to endothelial cells is considered a major contributor to the loss of microvascular density following AKI (1), most experimental studies on renal IR injury have focused on ischemic injury to tubular epithelial cells, while little has been published on early ischemic injury to endothelial cells. Our results show that mitochondrial swelling and loss of cristae membranes represent an early event in endothelial cells during ischemia, and the loss of cristae membranes limits the ability of these cells to regenerate ATP upon reperfusion as all respiratory chain complexes are localized on the inner mitochondrial membrane (6). We recently reported that SS-31 protected mitochondrial structure in proximal tubular cells during ischemia and accelerated the recovery of all ATP-dependent processes upon reperfusion, including cell-cell contact, cell attachment and restoration of the brush border (28). We now provide ultrastructural evidence that SS-31
also protects mitochondrial structure in capillary endothelial cells and prevents endothelial cell swelling, cell detachment and cell death. As a result, SS-31 protected endothelial barrier function and reduced interstitial edema and red blood cell extravasation. SS-31 also minimized “no-reflow” during reperfusion by inhibiting endothelial cell activation and prevented intravascular stasis and blockage of blood flow caused by platelet and leukocyte adhesion. In addition, SS-31 protected mitochondria in the medullary tubular cells, thereby preventing tubular cell swelling and vascular congestion (48). These ultrastructural findings help shed light on how SS-31 can reduce “no-reflow” in the heart after acute ischemic injury (28).

“No-reflow” prolongs ischemia time and aggravates endothelial cell degeneration and eventually leads to microvascular rarefaction (2, 3, 20, 21, 23, 25). Consistent with published reports (2, 23, 49, 52), we found a dramatic loss of glomerular and peritubular capillaries and cortical arterioles 4 weeks after acute ischemia. This microvascular loss was significantly mitigated by SS-31 treatment, and this was confirmed by the preservation of eNOS expression. Microvascular rarefaction leads to chronic tissue hypoxia with upregulation of proangiogenic factors such as VEGF (12, 33). The upregulation of VEGF was significantly reduced by SS-31 treatment, consistent with protection of the microvasculature. To the best of our knowledge, SS-31 is the first pharmacological intervention shown to protect endothelial mitochondria from acute ischemic injury and minimize subsequent microvascular loss and chronic tissue hypoxia. SS-31 has also been reported to promote the recovery of cortical arterioles in chronic renal ischemia caused by renal artery stenosis (14).
Hypoxia is a potent stimulus of inflammation and interstitial macrophage numbers and chronic damage were found to correlate with capillary density in human chronic kidney disease (24). We found a 23-fold increase in TNFα expression at 4 weeks after ischemia, and 8-fold increase in infiltrating macrophages and lymphocytes. All of these inflammatory markers were significantly reduced by SS-31 treatment. The tubulointerstitial influx of inflammatory cells contribute to fibrosis by releasing profibrotic cytokines including TGF-β1, a multifunctional cytokine that regulates cellular proliferation and extracellular matrix production (35,36,42,43). In this study, a 2.5-fold increase in TGF-β1 expression was associated with a significant increase in αSMA+ myofibroblasts and collagen deposition in the interstitium at 4 weeks. By minimizing chronic inflammation, SS-31 completely blocked the upregulation of TGF-β1 and mitigated fibrotic changes at 4 weeks. Besides preventing fibrosis, inhibition of TGF-β1 expression also helps to minimize tubular atrophy and promote angiogenesis (33).

In summary, both endothelial and epithelial mitochondria undergo swelling and loss of cristae during ischemia which prevents prompt recovery of ATP upon reperfusion. This results in endothelial cell activation, tubular cell swelling, and “no-reflow” upon reperfusion (illustrated in Figure 9). Prolonged ATP depletion leads to both endothelial and tubular epithelial cell death, tubular atrophy, microvascular rarefaction and chronic tissue hypoxia. Cell death and chronic hypoxia results in upregulation of proinflammatory cytokines, such as TNFα, and infiltration of inflammatory cells in the interstitium. These inflammatory cells contribute to interstitial fibrosis by releasing TGF-β1. While most therapeutic interventions currently target these downstream inflammatory and fibrotic
events, SS-31 represents a novel therapeutic intervention that minimizes ischemic injury by protecting mitochondrial structure during ischemia to allow for rapid restoration of ATP upon reperfusion (47). By minimizing tubular and endothelial cell death, SS-31 is able to prevent subsequent interstitial inflammation and fibrosis.

The loss of cristae membranes results from cardiolipin peroxidation that has been reported to occur during ischemia (31, 32, 38). Cardiolipin is a phospholipid that is exclusively expressed on the inner mitochondrial membrane and is required for proper cristae formation and for stabilization of respiratory chain supercomplexes for more efficient electron transfer (27, 37, 39, 51). We recently reported that SS-31 selectively binds to cardiolipin via electrostatic and hydrophobic interactions (6). By interacting with cardiolipin, SS-31 prevents cardiolipin from converting cytochrome c into a peroxidase while protecting its electron carrying function (4, 5). As a result, SS-31 protects mitochondrial cristae structure to allow rapid restoration of ATP levels for cell survival. Our results show that SS-31 is highly effective against the multifactorial phenomenon of IR injury by protecting mitochondria of all cell types involved.

Chronic kidney disease affects 8% of the United States population and frequently progresses to organ failure and cardiovascular death. Yet there are no effective therapies for renal fibrosis. Current drug development efforts have largely focused on inhibiting inflammatory and fibrotic pathways (18). Endothelial protection has recently been suggested as a novel therapeutic target for mitigating the progression of AKI to CKD (20). Transplantation of endothelial progenitor cells has been attempted in animal models but they failed to engraft in medullary blood vessels (13). To the best of our knowledge, SS-31
is the first agent that has been shown to protect endothelial cells from ischemic injury, prevent “no-reflow”, and reduce microvascular rarefaction, interstitial inflammation and fibrosis. Interestingly, continuous treatment with SS-31 over the 4 weeks did not provide further protection, suggesting that protecting the microvasculature from acute ischemic injury is sufficient to block downstream tissue remodeling. Our results suggest that SS-31 may be beneficial in minimizing the risk of progression to chronic kidney disease following acute ischemic injury. Based on its mechanism of action, SS-31 is best suited for clinical situations where renal ischemia is anticipated, such as transplantation, shock, and major surgery such as coronary artery bypass graft surgery and aortic aneurysm repair. SS-31 may be helpful in improving graft survival in renal transplantation as IR injury is a major cause of delayed graft function and chronic graft dysfunction (15, 36). A clinical formulation of SS-31 (Bendavia™) is currently in clinical trials for cardiac and renal IR injury (8).
DISCLOSURES

The SS peptide described in this article has been licensed for commercial research and development to Stealth Peptides Inc, a clinical stage biopharmaceutical company, in which H.H.S. and the Cornell Research Foundation have financial interests. The Research Program in Mitochondrial Therapeutics at Weill Cornell Medical College was established with a gift from Stealth Peptides International, Inc.

AUTHOR CONTRIBUTIONS

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


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## Table 1

Renal function after acute ischemia

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<th>Time after ischemia</th>
<th>Serum Creatinine</th>
<th>BUN</th>
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<tr>
<td></td>
<td>Saline</td>
<td>SS-31</td>
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<tr>
<td>24 hours</td>
<td>2.90 ± 0.08</td>
<td>1.36 ± 0.30***</td>
</tr>
<tr>
<td>1 week</td>
<td>0.46 ± 0.07</td>
<td>0.34 ± 0.04</td>
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<tr>
<td>2 weeks</td>
<td>0.44 ± 0.05</td>
<td>0.36 ± 0.02</td>
</tr>
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<td>3 weeks</td>
<td>0.44 ± 0.02</td>
<td>0.36 ± 0.02</td>
</tr>
<tr>
<td>4 weeks</td>
<td>0.46 ± 0.04</td>
<td>0.37 ± 0.02</td>
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Data are presented as mean ± SE. Each group started with n=7. Two animals in the saline group died within 48 hrs, and so there were only 5 animals at the subsequent times.
Figure Legends

Figure 1. Endothelial cell damage during acute IR injury. Rats were subcutaneously treated with saline 30 min before occlusion of renal blood flow bilaterally for 45 minutes followed by 5 minutes reperfusion. Ultrastructural studies of kidney sections obtained after 45 minutes ischemia alone, or after 5 minutes reperfusion, were carried out using transmission electron microscopy. (A) Representative sections from the outer medulla of a saline-treated rat showing a peritubular capillary filled with RBCs (magnified x 5000). The two endothelial cells (E) lining the capillary both show large vacuoles in their cytoplasm. (B) The highlighted areas from (A) were examined under x 80,000 magnification and revealed rounded, swollen mitochondria (M) with very few cristae membranes. (C) In addition to endothelial cells (E), interstitial cells (IC) also show large swollen mitochondria devoid of cristae membranes. (D) Following 5 minutes reperfusion, some endothelial cells could be seen to begin detaching from the basement membrane (arrow), while others show degenerated changes (*) and detachment. (E) Some capillaries show damage of the capillary wall (arrow) with leakage and RBC extravasation into the interstitial space. A medullary tubule (T) can also be seen with swollen epithelial cells and rounded mitochondria. (F) Platelet aggregates (P) can be seen in some capillaries that completely obstruct capillary blood flow.

Figure 2. SS-31 prevents endothelial cell damage during acute IR injury. Rats were subcutaneously treated with SS-31 (2 mg/kg) 30 minutes before occlusion of renal blood flow bilaterally for 45 minutes. Kidney sections were obtained after 45 minutes ischemia
alone, or with 5 minutes reperfusion, and examined using transmission electron microscopy. (A) Representative section from the outer medulla showing a medullary tubule (T) surrounded by 5 capillaries filled with RBCs after 45 minutes ischemia (magnified x5000). The endothelial cells (E) did not show the vacuoles seen in the saline-treated animals. (B) Representative capillary showing an endothelial cell with cytoplasmic processes well-attached to the capillary wall and no swollen mitochondria (x 15,000). The highlighted area is shown magnified x 80,000 in (C). The mitochondrion (M) is elongated with both inner and outer mitochondrial membranes and well-preserved cristae membranes. (D) Representative image showing cytoplasmic processes of the endothelial cell (E) wrapped around the capillary wall (x 10,000). (E) The highlighted area in (d) is magnified x 30,000, showing elongated mitochondrial with cristae membranes (arrows). (F) Within 5 minutes of reperfusion, blood flow is recovered in the peritubular capillaries with attached endothelial cells, no platelet aggregates, and no interstitial edema or RBCs extravasation.

Figure 3. SS-31 prevents mitochondrial changes in medullary tubular epithelial cells during acute IR injury. Rats were subcutaneously treated with saline or SS-31 (2 mg/kg) 30 minutes before occlusion of renal blood flow bilaterally for 45 minutes. Kidney sections were obtained after 45 minutes ischemia alone, or with 5 minutes reperfusion, and examined using transmission electron microscopy. (A) A medullary tubular epithelial cell showing cytoplasmic swelling and mitochondria that are rounded with sparse cristae membranes (x 8000). The boxed area is magnified in (B), highlighting the swollen...
mitochondria (M) with loss of cristae membranes and loss of basal membrane invaginations (x 30,000). (C) A representative medullary tubule from a SS-31 treated rat (x 5000). Note the well-organized elongated mitochondria and the lack of cell swelling. The boxed area is magnified in (D) showing well-preserved mitochondrial cristae even after 45 minutes ischemia (x 30,000).

Figure 4. SS-31 prevents tubular cell swelling and renal medullary vascular congestion 1 hour after acute IR injury. Rats were subcutaneously treated with saline or SS-31 (2 mg/kg) 30 minutes before occlusion of renal blood flow bilaterally for 45 minutes. Kidney sections were obtained after 1 hour reperfusion following 45 minutes ischemia, and stained with hemotoxylin and eosin. Representative sections from the inner stripe of the outer medulla are shown from sham-operated kidney, saline-treated IR kidney and SS-31 treated IR kidney (Top panel, x200). Swollen tubular cells are prevalent in the saline-treated samples (as indicated by arrows), and greatly reduced in the SS-31 treated samples. Swollen tubular cells led to compression of the peritubular capillaries, causing erythrocyte trapping and blocking blood flow in saline-treated IR kidney compared to sham-operated kidney (Bottom panel, x600). By preventing tubular cell swelling, erythrocyte trapping was greatly reduced in the SS-31 treated samples.

Figure 5. SS-31 reduced renal microvascular rarefaction 4 weeks after IR injury. Rats were subcutaneously treated with saline or SS-31 (2 mg/kg) 30 minutes before occlusion of renal blood flow bilaterally for 45 minutes. Kidney sections were obtained 4 weeks after
the acute ischemic injury. (A) Ischemia dramatically reduced the density of peritubular capillaries as quantified by immunostaining for CD31, an endothelial cell biomarker. SS-31 treatment during ischemia significantly increased peritubular capillaries at 4 weeks (n=4 in each group. ANOVA, ***P<0.001, *P<0.05). (B) Ischemia significantly reduced the density of glomerular capillaries as quantified by CD31 immunostaining, and this was also minimized by SS-31 treatment (n=4 in each group. ANOVA, P<0.001, ***P<0.001). (C) Ischemia significantly reduced the number of cortical arterioles as quantified by α-SMA immunostaining, and this was also reduced by SS-31 treatment (n=4 in each group. ANOVA, P<0.001, ***P<0.001). (D) Expression of eNOS was significantly reduced by ischemia and the down-regulation was significantly attenuated by acute SS-31 treatment. Continuous treatment with SS-31 for 4 weeks did not provide any further protection. (n=4 in each group; ANOVA, P<0.001, **P<0.01; *P<0.05). (E) Expression of VEGF was significantly increased by ischemia and the upregulation was reduced by acute SS-31 treatment. Continuous treatment with SS-31 for 4 weeks did not provide any further protection. (n=4 in each group; ANOVA, **P<0.01; *P<0.05).

**Figure 6.** SS-31 treatment during ischemia prevented histopathological changes 4 weeks after ischemia. Rats were subcutaneously treated with saline or SS-31 (2 mg/kg) 30 minutes before occlusion of renal blood flow bilaterally for 45 minutes. Kidney sections were obtained 4 weeks after the acute ischemic injury. Representative sections from the renal cortex and outer stripe of the outer medulla (OSOM) were stained with PAS and are shown for sham-operated, saline-treated IR, and SS-31 treated IR kidneys. Note the
thickening of the glomerular and tubular basement membranes, and tubular dilation and tubular atrophy in the saline group. All histopathological findings were minimized in the SS-31 treated animals.

Figure 7. SS-31 treatment during ischemia reduced interstitial inflammation 4 weeks after IR injury. Rats were subcutaneously treated with saline or SS-31 (2 mg/kg) 30 minutes before occlusion of renal blood flow bilaterally for 45 minutes. Kidney sections were obtained 4 weeks after the acute ischemic injury. (A) Acute SS-31 treatment significantly minimized the upregulation of TNFα expression in the outer medulla. Continuous treatment with SS-31 for 4 weeks did not provide any further protection. (n=4 in each group. ANOVA, P<0.001, ***P<0.001, **P<0.01). (B) Ischemia dramatically increased interstitial macrophage infiltration in the OSOM as measured by CD68 staining, and this was greatly suppressed by SS-31 treatment during ischemia (n=4 in each group. ANOVA, P<0.001, ***P<0.001). (C) Ischemia also significantly increased CD3+ lymphocytes in the OSOM, and this was also significantly prevented by SS-31 treatment during acute ischemia (n=4 in each group. ANOVA, P<0.001, ***P<0.001).

Figure 8. SS-31 treatment during ischemia reduced tubulointerstitial fibrosis 4 weeks after IR injury. Rats were subcutaneously treated with saline or SS-31 (2 mg/kg) 30 minutes before occlusion of renal blood flow bilaterally for 45 minutes. Kidney sections were obtained 4 weeks after the acute ischemic injury. (A) SS-31 treatment completely blocked the upregulation of TGFβ1 in the outer medulla. (n=4 in each group. ANOVA,
(B) Ischemia significantly increased fibrosis in the outer medulla as measured by Masson trichrome staining. This was prevented by SS-31 treatment during ischemia (n=4 in each group. ANOVA, ***P<0.001). (C) Acute ischemia increased the number of α-SMA expressing cells in the interstitium, and this was prevented by SS-31.

**Figure 9.** A schematic illustrating how SS-31 prevents the progression from acute ischemic kidney injury to chronic kidney disease. Ischemic injury to endothelial and epithelial cells result in endothelial cell activation, tubular cell swelling, and “no-reflow” upon reperfusion. Prolonged ATP depletion leads to both endothelial and tubular epithelial cell death, tubular atrophy, microvascular rarefaction and chronic tissue hypoxia. Cell death and chronic hypoxia results in upregulation of proinflammatory cytokines, such as TNFα, and infiltration of inflammatory cells in the interstitium. These inflammatory cells contribute to interstitial fibrosis by releasing TGF-β1. SS-31 minimizes ischemic injury by protecting mitochondrial structure during ischemia to allow for rapid restoration of ATP upon reperfusion. By minimizing tubular and endothelial cell death, SS-31 is able to prevent downstream interstitial inflammation and fibrosis.
Figure 3
Figure 4

Sham  Saline IR  SS-31 IR

x200  x200  x200

x600  x600  x600
Figure 5

A. 

B. 

C. 

D. 

E.
Figure 6

Sham Cortex OSOM Saline SS31

Cortex

OSOM

x100 x100 x100

x100 x100 x100
Figure 7

A

TNFα

β-actin

***

**

ns

B

C

Sham

Saline

SS31

Pump

Sham

Saline

SS-31

x200

Lymphocytes (μHPF)

***

***

Sham

Saline

SS31

x200

***

***

Microphages (μHPF)
Figure 8

A. TGF-β1 and β-actin Western blot analysis. The graph shows the fold-change in TGF-β1 expression in Sham, Saline, SS31, and Pump groups.

B. Immunohistochemical staining for TGF-β1. The images are at x200 magnification. The bar graph compares the collagen stain fold change in Sham, Saline, SS31, and SS31 pump groups.

C. Additional images showing the tissue sections stained for TGF-β1 at x200 magnification.
Figure 9

- **Acute ischemia**
  - Tubular cell death
  - Tubular atrophy
  - Microvascular rarefaction
  - Chronic tissue hypoxia
  - Interstitial inflammation
  - TNF-α
  - TGF-β1
  - Fibrosis