A Mitochondrial Permeability Transition Pore Inhibitor Improves Renal Outcomes After Revascularization in Experimental Atherosclerotic Renal Artery Stenosis

Alfonso Eirin, Zilun Li, Xin Zhang, James D. Krier, John R. Woollard, Xiang-Yang Zhu, Hui Tang, Sandra M. Herrmann, Amir Lerman, Stephen C. Textor, Lilach O. Lerman

Abstract—Revascularization improves blood pressure but not renal function in most patients with atherosclerotic renal artery stenosis (ARAS), possibly related to injury incurred during renal reperfusion. Bendavia, a novel tetrapeptide that inhibits mitochondrial permeability transition pore opening, reduces apoptosis, oxidative stress, and ischemia-reperfusion injury in experimental models. However, its potential for improving renal response to revascularization of chronic ARAS is unknown. We hypothesized that adjunct Bendavia would improve renal structure and function after percutaneous transluminal renal angioplasty (PTRA). Pigs were treated after 6 weeks of ARAS or control with PTRA+stenting (or sham), adjacent continuous 4-hour infusion of Bendavia (0.05 mg/kg IV) or vehicle (n=7 each) during PTRA. Single-kidney renal blood flow and glomerular filtration rate were studied 4 weeks later and renal mitochondrial biogenesis, microvascular architecture, and injurious pathways evaluated ex vivo. Monocyte chemoattractant protein-1 levels rose after PTRA, suggesting inflammatory injury. Bendavia did not immediately affect inflammatory cytokine levels, yet 4 weeks later, stenotic kidney renal blood flow and glomerular filtration rate both improved (44.00±2.01% and 36.40±10.21%, respectively) in ARAS+PTRA+Bendavia compared with ARAS+PTRA+vehicle. Renal mitochondrial biogenesis was restored after PTRA+Bendavia, and microvascular rarefaction, apoptosis, oxidative stress, tubular injury, and fibrosis decreased. Infusion of Bendavia during PTRA preserved mitochondrial biogenesis, renal hemodynamics, and function, and attenuated tissue injury in swine ARAS. Thus, functional mitochondrial injury during renal reperfusion may sustain renal inflammatory injury and limit kidney recovery after PTRA. Potent antiapoptotic and antioxidant effects provide Bendavia a novel therapeutic potential for improving kidney outcomes after PTRA in experimental ARAS. (Hypertension. 2012;60:1242-1249.) ● Online Data Supplement

Key Words: Bendavia □ renovascular hypertension □ revascularization □ oxidative stress □ reperfusion injury

Atherosclerotic renal artery stenosis (ARAS) remains a major cause of renovascular hypertension, associated with cardiovascular morbidity and progression to chronic renal failure. Although endovascular stenting has been widely applied to restore vessel patency in ARAS, several trials failed to establish added benefit of renal artery revascularization for improving renal function beyond medical therapy. Accordingly, we have shown previously that percutaneous transluminal renal angioplasty (PTRA) and stenting in swine ARAS restores blood pressure, but not tissue integrity and glomerular filtration rate (GFR) beyond the stenotic lesion. These observations highlight the need for interventions adjunctive to PTRA to attenuate kidney injury in ARAS.

Abrupt restoration of renal blood flow (RBF) can accentuate tissue injury by upregulating inflammation, oxidative stress, or other injurious pathways. Inflammation plays a central role in the pathogenesis of experimental acute renal ischemia-reperfusion injury (IRI), which is characterized by infiltration and release of inflammatory signals. Additionally, increased production of reactive oxygen species (ROS) or depleted adenine nucleotide levels in IRI trigger opening of the renal mitochondrial permeability transition pore (mPTP), a high-conductance channel formed in the inner mitochondrial membrane in response to insults. This may in turn induce mitochondrial dysfunction, apoptosis, inflammation, fibrosis, and renal dysfunction. Whether a component of IRI participates in the tissue injury in ARAS during revascularization is unknown.

Adaptive responses that preserve mitochondrial function can blunt IRI-elicited injury. Mitochondrial biogenesis is important in the kidney and increases cellular aerobic metabolic capacity. It is characterized by a complex network of

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1242
transcription factors that regulate expression of mitochondrial proteins that modulate mitochondrial function, and attenuates renal inflammation and oxidative stress by upregulating antioxidant and anti-inflammatory mediators. Peroxisome proliferator–activated receptor-γ coactivator (PGC)-1α, the main regulator of this pathway, protects against IRI by promoting recovery of mitochondrial function, and stimulates secretion of vascular endothelial growth factor (VEGF) and other angiogenic factors during ischemia. Whether targeting this pathway during revascularization would attenuate poststenotic kidney injury in ARAS remains unknown.

Bendavia is a novel compound that inhibits mitochondrial permeability transition pore (mPTP) opening, attenuates apoptosis and myocardial IRI, and reduces experimental myocardial infarct sizes when administered before onset of reperfusion. Continuous treatment with Bendavia until 2 hours after renal reperfusion decreases tubular cell, dysfunction, apoptosis, and necrosis in rats. However, its capability to improve outcomes after restoration of blood flow in chronic renal ischemia is unknown. We hypothesized that Bendavia infusion during renal revascularization would decrease kidney apoptosis, inflammation, and fibrosis and improve renal function in experimental ARAS.

**Methods**

All experiments were approved by the Institutional Animal Care and Use Committee. Twenty-eight domestic female pigs were observed for 16 weeks (Figure S1A in the online-only Data Supplement). At baseline, animals were randomized as normal (n=7) or ARAS (n=21). Normal animals were fed normal pig chow and ARAS pigs a high-cholesterol diet, which induces functional compromise, inflammation, and fibrosis.

Six weeks later, ARAS pigs underwent unilateral renal artery stenosis, induced by placing a local-irritant coil in the main renal artery, whereas normal animals were sham operated. Additionally, a telemetry transducer implanted in the left femoral artery continuously measured mean arterial pressure for the 10 following weeks. Six weeks after induction of renal artery stenosis, animals were anesthetized, the degree of stenosis determined by angiography, and pigs treated with PTRA or sham (Figure S1B). Additionally, ARAS pigs were treated with a continuous intravenous infusion of Bendavia 0.050 mg/kg, or an equal volume of saline, from 30 minutes before to 3.5 hours after PTRA or sham. Inferior vena cava samples were collected for pharmacokinetic analysis, inflammatory, and injury biomarkers immediately before and 30 and 180 minutes postreperfusion and urinary samples before Bendavia infusion and 210 minutes after PTRA.

Four weeks later, the pigs were again anesthetized; inferior vena cava samples collected for plasma renin activity, creatinine, and cholesterol measurements; and urine samples for albumin concentration. Bilateral single-kidney hemodynamics, and function were assessed using multidetector computed tomography. Three days after completion of in vivo studies, animals were euthanized (sodium pentobarbital, 100 mg/kg) and kidneys dissected and prepared for ex vivo studies. Renal arteries from normal animals were harvested and suspended in organ chambers filled with Kreb solution to evaluate vascular reactive response to Bendavia.

Inferior vena cava levels of tumor necrosis factor (TNF)-α, interleukin-1β, monocyte chemotaxtractant protein (MCP-1), granulocyte colony-stimulating factor (G-CSF), and transforming growth factor (TGF)-β were measured by ELISA before and after PTRA. Serum creatinine levels, urine 8-epi-isoprostane, and proteinuria were measured by standard procedures.

Apoptosis was assessed in renal sections stained with terminal deoxynucleotidyl transferase dUTP nick end labeling and caspase-3 and protein expression of B-cell lymphoma (Bcl)-2 and Bcl-2-associated X-protein by Western blot. Mitochondrial biogenesis was evaluated by renal expression of PGC-1α, nuclear respiratory factor-1, GA-binding protein, peroxisome proliferator–activated receptor (PPAR)-α, PPAR-δ, heme oxygenase-1, and sirtuin-1. Microvascular architecture was assessed using 3D microcomputed tomography and angiogenesis by expression of VEGF and its receptors (VEGF-R1 and -2).

Renal scarring was assessed in kidney sections stained with Masson trichrome (tubulointerstitial fibrosis and glomerular score), fibronectin, collagen IV, and expression of plasmogen-activator inhibitor-1 and TGF-β (Western blot). Tubular injury was assessed in sections stained with periodic acid-Schiff and renal inflammation in sections stained with MCP-1 or CD163 and by TNF-α expression (Western blot). Oxidative stress was assessed by dihydroethidium staining, systemic levels of 8-epi-isoprostane, renal protein expression of the reduced nicotinamide-adenine dinucleotide-oxidase subunit p47, and nitrotyrosine.

Contralateral kidney (CLK) inflammation (MCP-1), oxidative stress (dihydroethidium), apoptosis (terminal deoxynucleotidyl transferase dUTP nick end labeling), and fibrosis (Trichrome) were also assessed.

**Statistical Methods**

Results were expressed as mean±SD or medium (range). Parametric (ANOVA/Student t test) and nonparametric (Wilcoxon/Kruskal-Wallis) tests were used as appropriate. P<0.05 was considered statistically significant. For detailed Methods and Results, see the online-only Data Supplement.

**Results**

Immediately before revascularization, significant renal artery stenosis was achieved in all ARAS animals (77.5% [65.0–95.0%]), and mean arterial pressure was similarly elevated compared with controls (Figure S1B).

**Bendavia and Injury Signals During PTRA**

Systemic plasma Bendavia concentration increased to therapeutic levels (≥100–200 ng/mL) 30 minutes postinfusion, reaching an apparent steady state concentration at 60 to 90 minutes (Table 1 in the online-only Data Supplement). Systemic MCP-1 levels increased after revascularization similarly in ARAS+PTRA+vehicle and ARAS+PTRA+Bendavia (Figure S2A, P<0.05 versus baseline). Systemic levels of TNF-α, interleukin-1β, G-CSF, TGF-β, and creatinine remained unchanged (Figure S2B through S2F), as did urinary protein and 8-epi-isoprostane levels (Figure S3A and S3B).

**Outcomes After PTRA**

PTRA-treated animals showed no residual stenosis, and mean arterial pressure decreased to normal levels 4 weeks after PTRA (Figure S1B). Serum creatinine remained elevated in all ARAS groups (Table 1). Plasma renin activity and urinary albumin levels remained unchanged, whereas cholesterol levels were higher in all ARAS compared with normal (Table S1). Four weeks after revascularization, stenotic kidney RBF and GFR were reduced in ARAS, unchanged by PTRA, but restored in ARAS+PTRA+Bendavia pigs (Table 1).

**Bendavia Decreased Apoptosis and Promoted Mitochondrial Biogenesis**

The numbers of cells positive for terminal deoxynucleotidyl transferase dUTP nick end labeling and caspase-3 were
Table 1. Systemic and Renal Characteristics of Pig Group (n=7 each) After PTRA or Sham

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal</th>
<th>ARAS</th>
<th>ARAS+PTRA+Vehicle</th>
<th>ARAS+PTRA+Bendavia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean arterial pressure, mm Hg</td>
<td>85.9±2.8</td>
<td>166.6±2.4**</td>
<td>90.6±2.8</td>
<td>106.8±2.9</td>
</tr>
<tr>
<td>Serum creatinine, mg/dL</td>
<td>1.45±1.52</td>
<td>1.96±0.30*</td>
<td>1.91±0.43*</td>
<td>1.98±0.36*</td>
</tr>
<tr>
<td>8-epi-isoprostane, pg/mL</td>
<td>94.3±12.5</td>
<td>133.2±11.2†</td>
<td>136.9±13.3†</td>
<td>118.2±13.9</td>
</tr>
<tr>
<td>Stenotic kidney RBF, mL/min</td>
<td>652.1±23.7</td>
<td>394.0±30.7†</td>
<td>425.7±48.2†</td>
<td>613.2±48.3</td>
</tr>
<tr>
<td>Stenotic kidney GFR, mL/min</td>
<td>82.9±8.8</td>
<td>51.4±3.8**</td>
<td>49.7±4.9**</td>
<td>67.8±4.4</td>
</tr>
</tbody>
</table>

*ARAS indicates atherosclerotic renal artery stenosis; PTRA, percutaneous transluminal renal angioplasty; RBF, renal blood flow; GFR, glomerular filtration rate.

**P<0.05 vs normal.
†P<0.05 vs ARAS+PTRA+Bendavia.

Elevated in ARAS and ARAS+PTRA+vehicle compared with normal. Bendavia-treated animals had fewer positive cells than other ARAS groups, yet remained elevated (Figure 1A and Figure S4A). Renal expression of Bcl-2 was unchanged, but Bendavia reduced subsequent expression of the proapoptotic Bcl-2-associated X-protein (Figure 1B).

Furthermore, Bendavia upregulated expression of PGC-1α, nuclear respiratory factor-1, GA-binding protein, and PPAR-α (Figure 1B), whereas heme oxygenase-1 and PPAR-β remained blunted. Revascularization uniformly restored the downregulated expression of sirtuin-1 in ARAS (Figure 1B).

**Bendavia Increased Microvascular Density**

Spatial density of renal cortical microvessels was similarly low in ARAS and ARAS+PTRA+vehicle animals but not different from normal in ARAS+PTRA+Bendavia (Figure 1C). In addition, the increase in vessel diameter and tortuosity observed in ARAS and ARAS+PTRA+vehicle was normalized in Bendavia-treated pigs (Figure 1C). Renal expression of VEGF and its receptors (VEGFR-1 and R2) was attenuated in ARAS and ARAS+PTRA+vehicle, but PTRA+Bendavia restored them to levels similar to or above normal (Figure 1D).

**Bendavia Decreased Oxidative Stress and Inflammation**

Systemic levels of 8-epi-isoprostane were elevated in ARAS and ARAS+PTRA+vehicle but decreased in ARAS+PTRA+Bendavia pigs (Table 1). Similarly, in situ production of superoxide anion in the poststenotic kidney decreased in ARAS+PTRA+Bendavia (Figure 1E), as did expression of p47 and nitrotyrosine (Figure 2A).

MCP-1 immunoreactivity was equally upregulated in ARAS and ARAS+PTRA+vehicle but ameliorated in ARAS+PTRA+Bendavia, as were the number of CD163+ macrophages (Figure 2B and 2C and Figure S4B). Similarly, elevated renal expression of TNF-α was normalized in Bendavia-treated pigs (Figure 2D).

**Bendavia Decreased Tubular Damage and Renal Scarring**

Tubular injury score, tubulointerstitial fibrosis, and glomerular score were elevated in all ARAS groups (P<0.05 all), but blunted in ARAS+PTRA+Bendavia (Figure 2E and 2F). Immunostaining of profibrotic fibrinectin and collagen IV was also reduced in Bendavia-treated pigs (Figure 2G and Figure S4C). Renal expression of plasminogen-activator inhibitor-1 was attenuated by PTRA, but TGF-β1 expression was restored only in Bendavia-treated pigs (Figure 2H).

**Effect of Bendavia on the CLK**

CLK RBF and GFR did not differ among the groups (Figure S5). The number of apoptotic cells was elevated in the CLK of ARAS and ARAS+PTRA+vehicle but preserved in ARAS+PTRA+Bendavia (Figure S6). CLK superoxide-anion production (Figure S7) and MCP-1 expression (Figure S8) were similarly elevated in all ARAS compared with normal. Tubulointerstitial fibrosis was also elevated in ARAS but attenuated in all PTRA-treated pigs (Figure S9). Incidentally, glomerular score was lower in ARAS+PTRA+vehicle compared with ARAS and ARAS+PTRA+Bendavia (Figure S10).

**Discussion**

This study demonstrates that reperfusion by PTRA in swine ARAS acutely increased cytokine levels (MCP-1), associated 4 weeks later with multiple markers of inflammatory injury, oxidative stress, apoptosis, and interstitial fibrosis. Systemic administration during revascularization of Bendavia, an mPTP-targeted peptide, attenuated adverse renal responses to revascularization, suggesting a novel role for mitochondrial-targeted therapies for limiting reperfusion injury.

Four weeks after revascularization, mitochondrial biogenesis was upregulated in ARAS+PTRA+Bendavia poststenotic kidneys, whereas oxidative stress, apoptosis, microvascular loss, and tissue injury were ameliorated. Hence, IRI during PTRA may limit recovery in experimental ARAS. Furthermore, stenotic kidney RBF and GFR were normalized in Bendavia-treated ARAS pigs, consistent with renoprotective effects of Bendavia in conjunction with PTRA.

ARAS activates multiple mechanisms that increase oxidative damage, apoptosis, inflammation, and interstitial fibrosis, leading to progressive renal dysfunction. However, controlled clinical trials dissociate restoring renal artery patency and subsequent renal outcomes. We have shown that PTRA alone fails to reverse structural and functional deterioration in the stenotic swine kidney, underscoring the need for effective strategies in combination with PTRA to improve renal function in ARAS.
Figure 1. A, Quantification of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and caspase-3 apoptotic cells. B, Renal protein expression of B-cell lymphoma (Bcl)-2, Bcl-2-associated X-protein (Bax), peroxisome proliferator–activated receptor-γ coactivator (PGC)-1α, nuclear respiratory factor (NRF)-1, GA-binding protein (GABP), peroxisome proliferator–activated receptor (PPAR)α, PPAR-δ, heme oxygenase (HO)-1, and sirtuin (SIRT)-1 relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). C, Representative 3D microcomputed tomography images (top), spatial density, diameter, and tortuosity (bottom) of cortical microvessels. D, Renal expression of vascular endothelial growth factor (VEGF) and its receptors (VEGFR-1 and VEGFR-2). E, Renal production and quantification of superoxide anion (dihydroethidium [DHE], ×40). *P<0.05 vs normal; †P<0.05 vs atherosclerotic renal artery stenosis (ARAS)+percutaneous transluminal renal angioplasty (PTRA)+Bendavia.
Figure 2. A. Renal protein expression of p47 and nitrotyrosine (NT) relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were normalized in Bendavia-treated pigs. B. Monocyte chemoattractant protein (MCP)-1 staining and (C) CD163+ cells per field. D. Renal expression of tumor necrosis factor (TNF)-α. E. Representative periodic acid-Schiff staining (left, purple-magenta) and quantification of tubular injury (right). F. Representative tubulointerstitial trichrome staining (left) and quantification of tubulointerstitial fibrosis and glomerular score (right). G. Quantification of staining with fibronectin and collagen IV. H. Renal expression of plasminogen-activator inhibitor (PAI)-1 and transforming growth factor (TGF)-β was normalized in Bendavia-treated animals. *P<0.05 vs Normal; †P<0.05 vs atherosclerotic renal artery stenosis (ARAS)+percutaneous transluminal renal angioplasty (PTRA)+Bendavia.
Our results implicate in the mechanisms that perpetuate renal damage during PTRA a form of IRI involving sustained activation of inflammation and subsequent ROS production.\(^6\) mPTP formation is a primary pathway accelerating cell death after IRI.\(^6\) Excessive ROS production leads to mPTP opening and release of cytochrome-C into the cytosol, which triggers not only apoptosis (by activating caspase-3 and -9) but also tubular damage via release of mitochondrial ROS into the cytosol.\(^6\) Hence, therapeutic interventions that selectively target the mPTP confer cytoprotection and mitigate progression to renal failure.

Previous studies in animal models have documented a protective effect of the mitochondrial-targeted Bendavia by attenuating apoptosis and oxidative stress.\(^16,17\) In addition to antiapoptotic effects,\(^18\) the ability of Bendavia to prevent ROS formation by improving the efficacy of the electron-transport chain along the inner mitochondrial membrane provides a unique potency for inhibiting oxidative stress compared with conventional antioxidant therapies.\(^19\) Indeed, cyclosporine (a potent mPTP inhibitor) treatment immediately before coronary revascularization decreases infarct size in patients with acute myocardial infarction,\(^20\) underscoring the potential clinical importance of such interventions.

Bendavia is a cell-permeable tetrapeptide that reaches high concentrations in the inner mitochondrial membrane,\(^21\) exerting both antiapoptotic and antioxidant properties. Unlike other mPTP inhibitors like cyclosporine, Bendavia does not suppress the activity of PPAR-\(\alpha\), GA-binding protein, and PPAR-\(\alpha\). Bendavia administration reduces infarct size\(^12\) and attenuated hypertensive cardiomyopathy after Bendavia administration.\(^20\) Moreover, it prevents interstitial fibrosis and accelerated tubular regeneration in rat acute IRI\(^13\) and ureteral obstruction.\(^20\) Our results extend these observations to indicate that adjunct Bendavia during PTRA restores mitochondrial biogenesis and attenuates apoptosis and oxidative stress, leading to improved renal function in chronic swine ARAS. The antiapoptotic effect of Bendavia was reflected by reduced number of terminal deoxynucleotidyl transferase dUTP nick end labeling-positive and caspase-3-positive cells. Furthermore, renal expression of the proapoptotic protein Bcl-2-associated X-protein was substantially decreased in Bendavia-treated pigs, underscoring the effectiveness of therapies oriented to prevent initiation of apoptosis. Additionally, PTRA+Bendavia reduced both systemic and renal oxidative stress, evidenced by decreased 8-isoprostanes, ROS production, and expression of reduced nicotinamide-adenine dinucleotide-oxidase (p47phox) and nitrotyrosine.

This might have also contributed to preservation of the stenotic kidney microvascular network. Augmented or prolonged production of ROS compromises the integrity of renal microvessels, leading to remodeling or rarefaction,\(^15\) important determinants of the progression of renal injury.\(^21\) In the current study, cortical microvascular loss was blunted in ARAS+PTRA+Bendavia pigs, reflected in their increased spatial density and decreased diameter, whereas tortuosity, reflecting microvascular immaturity, decreased. Bendavia might have also prevented microvascular loss by blunting vascular cell apoptosis and promoting angiogenesis, suggested by increased renal expression of VEGF and its receptors.

Renal inflammation is a critical determinant of disease progression in ARAS. Increased oxidative stress is associated with macrophage and lymphocyte infiltration into the stenotic swine kidney\(^24\) and poor renal responses to revascularization.\(^25–27\) A major new finding in this study is that short-term treatment with Bendavia during PTRA abolished stenotic kidney inflammation 4 weeks later, as evidenced by normalized expression of the proinflammatory cytokines TNF-\(\alpha\) and MCP-1, possibly accounting for reduced infiltration of CD163 macrophages. Importantly, reduced inflammation, oxidative stress, and apoptosis after Bendavia treatment were all associated with attenuated tubular damage. Indeed, in rats with acute IRI, Bendavia preserved tubular brush borders and minimized tubular cell detachment.\(^13\)

Importantly, Bendavia seemed to promote mitochondrial biogenesis, as defined by restored levels of PGC-1\(\alpha\), NRF-1, GA-binding protein, and PPAR-\(\alpha\), implicating this pathway in amelioration of oxidative stress and inflammation. PGC-1\(\alpha\)-mediated activation of nuclear respiratory factor-1 and GA-binding protein (nuclear respiratory factor-2) in turn regulates expression of genes involved in oxidative stress. Furthermore, Bendavia-induced mitochondrial biogenesis upregulated expression of PPAR-\(\alpha\), a transcription factor that regulates renal macrophage and endothelial cell inflammatory responses by augmenting heme oxygenase-1 enzymatic activity,\(^28\) although heme oxygenase-1 expression remained suppressed. Similarly, renal expression of PPAR-\(\beta\) and sirtuin 1 was uniformly restored after PTRA, suggesting their contribution to attenuate inflammation after Bendavia. Taken together, these observations suggest that adjunctive infusion of Bendavia during PTRA prevented an acute fall in mitochondrial biogenesis, which might have accounted for the improved renal function and structure sustained 4 weeks later.

An additional observation was that treatment with PTRA+Bendavia decreased tubulointerstitial and glomerular fibrosis 4 weeks later, associated with decreased fibronectin and collagen IV content. Furthermore, renal expression of the fibrogenic factors TGF-\(\beta\) and plasminogen-activator inhibitor-1 was normalized in Bendavia-treated animals, possibly related to decreased renal oxidative stress. In turn, these might have contributed to improved renal function, evidenced by normalized single-kidney RBF and GFR, underscoring the feasibility of Bendavia to attenuate renal dysfunction in ARAS. These effects are unlikely because of the direct regulation of vascular tone, given the lack of renal vascular reactivity response to Bendavia in vitro (Figure S3C and S3D). Despite improvement in stenotic kidney GFR, serum creatinine levels remained unchanged, possibly because of residual hypertensive injury in the CLK (persistent oxidative stress, inflammation, and glomerulosclerosis). These observations argue against major involvement of the mPTP in acute CLK injury during PTRA in swine ARAS.

Notably, MCP-1 levels increased within 3 hours after PTRA, suggesting inflammatory IRI, in agreement with studies in rats showing a progressive increase in renal expression of MCP-1 immediately after reperfusion.\(^29\) We did not detect
major changes in other inflammatory or oxidative mediators immediately after revascularization. Although the increase in MCP-1 levels was unaffected by the drug, Bendavia might have inhibited IRI mediators downstream to MCP-1 during PTRA. Furthermore, we cannot exclude plausible changes in intracellular and mitochondrial oxidative stress or inflammation related to reperfusion that were not evident in systemic samples, which would be consistent with concentration of Bendavia within mitochondria. Bendavia is usually cleared in the urine within 36 to 48 hours of administration (unpublished data), yet its plasma levels may increase in subjects with decreased renal clearance and prolong its protective effects. Evidently, exposure to the drug during PTRA in our studies was sufficient to confer potent protective effects measured 4 weeks later.

The limitations of this study included the use of young animals, short duration of the disease, and lack of additional comorbidities. Possibly, collection of additional blood samples in the first few days after PTRA would have allowed detection of further changes in renal function or inflammatory/oxidative mechanisms related to IRI. Further studies are needed to examine whether the observed effects of Bendavia are sustained longer term. Nevertheless, this study has a number of strengths. Our swine model reproduces the effects of early atherosclerosis and allows studying single-kidney function and structure using clinically applicable tools, offering the opportunity to assess the potential effects of Bendavia in the ARAS kidney.

Perspectives

Our results underscore the importance of ongoing inflammatory and profibrotic injury that PTRA alone fails to reverse in experimental ARAS, despite reducing arterial pressure. The rise in MCP-1 immediately after PTRA suggests a reperfusion injury analogous to IRI that may prevent recovery of kidney function. Our observations establish that infusion at the time of PTRA of Bendavia, a compound known to localize within mitochondria and modulate mPTP, decreases apoptosis, inflammation, and oxidative stress in swine ARAS. This maneuver improved mitochondrial biogenesis, angiogenesis, and ultimately renal function after revascularization. These results support the premise that reperfusion injury mediated by mitochondrial transition pore dynamics may be an important target for improving responses after PTRA in chronic renovascular disease.

Sources of Funding

This study was supported by a grant from Stealth Peptides, Inc.

Disclosures

None.

References


What Is New?
• Our study implicates mitochondrial permeability transition pore opening and downregulated mitochondrial biogenesis in the inconsistent effectiveness of revascularization for restoring renal structure and function in atherosclerotic renal artery stenosis.

What Is Relevant?
• The failure of revascularization to improve renal function in atherosclerotic renal artery stenosis is a recognized dilemma for patient management.

Novelty and Significance

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Summary
• Bendavia, a mitochondrial permeability transition pore opening inhibitor, shows a unique therapeutic potential for improving kidney outcomes after revascularization in chronic experimental atherosclerotic renal artery stenosis.

Our study proposes a novel and clinically applicable therapeutic option to improve the efficacy of percutaneous transluminal renal angioplasty.
ONLINE SUPPLEMENTAL MATERIAL

A mitochondrial permeability transition pore inhibitor improves renal outcomes after revascularization in experimental atherosclerotic renal artery stenosis

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Methods

In-vivo studies:
Animals were induced with an intramuscular ketamine and xylazine (0.5g), and anesthesia maintained with intravenous ketamine (0.2mg/kg/min) and xylazine (0.03mg/kg/min). MDCT studies were performed for assessment of single-kidney renal hemodynamics and function. Following a central venous injection of iopamidol (0.5mL/kg per 2 seconds), 140 consecutive scans were performed. Cross-sectional images were reconstructed, and analyzed with the Analyze™ software package (Biomedical Imaging Resource, Mayo Clinic, Rochester, MN). Cortical and medullary volume and perfusion, renal blood flow (RBF), and GFR were calculated, as described in detail previously1-5.

Ex-vivo studies:
Pharmacokinetic analysis
4 mL venous whole blood samples were collected from Bendavia-treated animals at baseline (immediately before Bendavia infusion), immediately before PTRA, and 30 and 180 min post-reperfusion. Venous blood were drawn using syringes into BD Vacutainer® PST™ Plasma Separation Tubes (lavender top) containing K2EDTA, 10 mL/tube. Tubes were gently inverted 8 times and kept in an ice water bath until centrifugation. Within an hour samples were centrifuged in a swing bucket centrifuge at 1000-1300 RCF (or approximately 1500xG) for 15 min at 4°C. Plasma were harvested from individual blood tubes, placed in a single polypropylene vials (or screw-cap tubes) and stored at approximately -80°C until assay.

Bendavia (MTP-131) concentration in pig plasma was determined by QPS (Newark, DE) using a qualified LC/MS/MS assay in K2-EDTA pig plasma. The assay employed deuterium labeled d5-MTP-131 as the analytical internal standard (IS). In brief, samples were spiked with internal standard, processed by protein precipitation extraction (recovery approximately 90%), and analyzed using reversed-phase HPLC with Turbo Ion Spray®MS/MS detection. Positive (M+2H)^2+ ions for MTP-131 and the IS (d5-MTP-131) were monitored in MRM mode. Drug-to-IS peak area ratios for the standards were used to create a linear calibration curve ranging from 2.5 to 1000ng/mL. The inter-day coefficient of variation for assay precision was less than 10%, and the accuracy ranged from 3.6 to 11.8%.

Inflammatory and injury markers
IVC levels of tumor necrosis factor (TNF)-α (Invitorgen, Cat# KSC3011), interleukin (IL)-1β (R&D systems DY681), monocyte chemoattractant protein (MCP-1) (Kingfisher Biotech, Cat# VS0081S-002), granulocyte colony-stimulating factor (G-CSF) (NovaTein Bio. Cat# BG-POR11157), and transforming growth factor (TGF)-β were measured by Enzyme-linked immunosorbent assay (ELISA) at baseline (before Bendavia infusion) and 30min and 180min after PTRA. Similarly, serum creatinine levels (baseline, 30min, and 180min post PTRA) as well as urinary levels of 8-epi-isoprostane, and proteins were measured by standard procedures at baseline and 210min after PTRA.
Apoptosis and mitochondrial biogenesis
Apoptosis was assessed in renal tissue sections stained with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL, Promega, Madison, WI, USA) and caspase-3 (1:200, Santa Cruz). In addition, renal protein expression of the apoptosis regulator proteins B-cell lymphoma (Bcl)-2, Lifespan BioSciences, 1:1000) and Bcl-2-associated X protein (Bax, Santa Cruz, 1:200) was evaluated by Western blot. Mitochondrial biogenesis was evaluated by renal expression of PGC-1α (Abcam, 1:1000), nuclear respiratory factor (NRF)-1 (Abcam, 1:300), GA-binding protein (GABP) (Abcam, 1:1000), peroxisome proliferator-activated receptor (PPAR)-α (Abcam, 1:1000), PPAR-δ (Abcam, 1:300), Heme oxygenase (HO)-1 (Abcam, 1:250), and sirtuin (SIRT)-1 (Abcam, 1:1000).

Microvascular architecture and angiogenesis
To evaluate microvascular architecture, kidneys were perfused under physiologic perfusion pressure with a radio-opaque silicone polymer (Microfil MV122; Flow Tech, Carver, MA). Then, perfused kidney sections were scanned using a micro-CT scanner, and reconstructed images (18µm voxels) displayed using Analyze™. Spatial density, average diameter, and tortuosity of renal cortical microvessels (diameters of 40–500µm) were calculated, as previously described6. In addition, renal protein expression of VEGF and its receptors (VEGFR-1 and 2) (Santa Cruz 1:200) was measured by Western blot.

Renal morphology and fibrosis
Renal fibrosis was assessed in 5-µm mid-hilar cross-sections of each kidney stained with Masson's trichrome by using the computer-aided image-analysis program AxioVision® 4.8.2.0 (Carl ZEISS SMT, Oberkochen, Germany). Tubulo-interstitial fibrosis and glomerular score (% of sclerotic out of 100 glomeruli) were quantified in 15–20 fields7, 8. In addition, tubular injury was scored in a blinded fashion in sections stained with Periodic acid-Schiff (PAS)9. Briefly, tubular injury (dilation, atrophy, cast formation, cell detachment, or thickening of tubular basement membrane) was scored from 1 to 5, 0 being normal tubules, 1: <10% of tubules injured, 2: 10-25% of tubules injured, 3: 26-50% of tubules injured, 4: 51-75% of tubules injured, 5: >75% of tubules injured.

Inflammation and oxidative stress
Renal inflammation was assessed in tissue sections stained with MCP-1 or CD163 (quantification of renal macrophages), and by protein expression of TNF-α (Santa Cruz 1:200) measured by Western blot10, 11. Oxidative stress was assessed by dihydroethidium (DHE) staining of kidney tissue, systemic levels of 8-epi-isoprostane (EIA kit)12, renal protein expression of the NADPH-oxidase sub-unit p47 (Santa Cruz 1:200), and nitrotyrosine (Cayman Chemical 1:200)12, 13.

Vascular reactivity
The vasodilator reactivity of Bendavia was evaluated, as previously described for coronary 14-16 and renal17, 18 specimens. Dissected renal artery sections (2-3mm long, 2 per animal) were suspended in 25ml organ chambers filled with Kreb’s solution at 37°C.
(pH=7.4, 95% O₂, and 5% CO₂). Isometric force was measured by suspending renal artery sections using 2 stainless clips passed through their lumen attached to a stationary post and a strain gauge. By using potassium chloride (KCl, 20mM) vessel rings were progressively stretched to achieve the optimal point for their length-tension relationship.

Once optimal tension was determined vessel rings were allowed to equilibrate for 30 minutes after washing with control solution. In 4 vessel rings, increasing doses of Bendavia (10⁻⁹ M to 10⁻⁴ M) were administered to test for the presence of vasoconstrictor response. In the other 4 rings, increasing doses of Bendavia were administered following precontraction with endothelin-1 (10⁻⁷ M) (Phoenix Pharmaceuticals, Mountain View, CA), to evaluate endothelial relaxation. Data was quantified using WinDaq Acquisition Software (DATAQ Instruments, Inc. Akron, OH).

**Statistical methods**

All data were analyzed using JMP software package version 8.0 (SAS Institute Inc. Cary, NC). The Shapiro-Wilk test was used to test for deviation from normality. Results were expressed as mean ± standard deviation (SD) for normally distributed data, and medium (range) for non-normally distributed data. Parametric (ANOVA and unpaired Student t-test) and non-parametric (Wilcoxon and Kruskal Wallis) tests were used as appropriate. Values of p≤0.05 were considered statistically significant.

**Results**

**Pharmacokinetic analysis**

Bendavia IV infusion started at 30 minutes prior to the PTRA procedure and continued for 210 minutes at 0.05 mg/kg/h. Mean plasma concentration at 30 minutes into infusion (i.e. time of PTRA procedure) was 100.6ng/mL (Table S1). Plasma concentration continued to increase, reached an apparent steady state concentration (~125 ng/mL) at approximately 60-90 minutes, and maintained a steady state level through end of infusion.

**Inflammatory and injury markers during PTRA**

Plasma levels of monocyte chemoattractant protein (MCP-1) similarly increased after revascularization in ARAS+PTRA+Vehicle and ARAS+PTRA+Bendavia animals (Figure 1B, both p<0.05 vs. baseline). However, plasma levels of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, granulocyte colony-stimulating factor (G-CSF), transforming growth factor (TGF)-β, or serum creatinine levels did not change immediately after revascularization (Figure S2B-F).

Proteinuria as well as urinary levels of 8-isoprostanes and TGF-β remained unchanged after PTRA (Figure S3A-B).

**Vascular reactivity**

Isolated renal artery rings did not respond to increasing doses of Bendavia (Figure S3D-E), suggesting that it induces neither vasoconstriction nor vasodilation.
References


Table S1: Mean plasma Bendavia (MTP-131) concentration during a 3.5-h IV infusion (0.05mg/kg/h) in a swine model of atherosclerotic renal artery stenosis.

<table>
<thead>
<tr>
<th>Time from PTRA (min)</th>
<th>Time from Bendavia Infusion (min)</th>
<th>Mean Bendavia Concentration (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-30</td>
<td>0</td>
<td>BQL</td>
</tr>
<tr>
<td>PTRA 30</td>
<td>30</td>
<td>100.6±36.2</td>
</tr>
<tr>
<td>30</td>
<td>60</td>
<td>118.4±23.7</td>
</tr>
<tr>
<td>60</td>
<td>90</td>
<td>125.1±21.4</td>
</tr>
<tr>
<td>180</td>
<td>210</td>
<td>111.3±42.0</td>
</tr>
</tbody>
</table>

PTRA: percutaneous transluminal renal angioplasty; BQL: below quantifiable limit (2.5ng/mL); NA: not applicable.
Table S2: Systemic and renal characteristics of normal, ARAS, ARAS+PTRA+Vehicle, and ARAS+PTRA+Bendavia pigs (n=7 each) 4 weeks after PTRA or sham.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NORMAL</th>
<th>ARAS</th>
<th>ARAS+PTRA+Vehicle</th>
<th>ARAS+PTRA+Bendavia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (Kg)</td>
<td>46.1±2.1</td>
<td>44.7±1.8</td>
<td>43.0±1.3</td>
<td>46.7±2.2</td>
</tr>
<tr>
<td>Degree of stenosis (%)</td>
<td>0</td>
<td>88.7±3.9*†</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Plasma renin activity (ng/ml/hr)</td>
<td>0.12 ± 0.05</td>
<td>0.16 ± 0.13</td>
<td>0.15 ± 0.09</td>
<td>0.14 ± 0.11</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>88.0 (74-122)</td>
<td>519.0 (411-592)*</td>
<td>492.0 (229-823)*</td>
<td>446.5 (97-761)*</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>7.0 (5-10)</td>
<td>6.0 (4-28)</td>
<td>6.0 (1-8)</td>
<td>7.0 (4-11)</td>
</tr>
<tr>
<td>Low-density-lipoprotein (mg/dl)</td>
<td>47.6 (33-58)</td>
<td>324.6 (174-392)*</td>
<td>264.8 (114-332)*</td>
<td>295.8 (33-409)*</td>
</tr>
<tr>
<td>Urinary albumin (μg/ml)</td>
<td>4.0±2.6</td>
<td>3.8±1.1</td>
<td>3.3±3.0</td>
<td>3.8±3.3</td>
</tr>
</tbody>
</table>

*p≤0.05 vs. Normal; †p<0.05 vs. ARAS+PTRA+Bendavia.
Figure S1. Schematic of the experimental protocol showing interventions and time points (A). Evolution of mean arterial pressure measured using telemetry (B).
Figure S2. Inferior vena cava levels of monocyte chemoattractant protein (MCP-1), tumor necrosis factor (TNF)-α (B), interleukin (IL)-1β (C), granulocyte colony-stimulating factor (G-CSF) (D), transforming growth factor (TGF)-β (E), and serum creatinine (Scr) (F) in ARAS+PTRA+Vehicle and ARAS+PTRA+Bendavia pigs at baseline (before Bendavia infusion), and 30 and 180min after PTRA. #p<0.05 vs. baseline.
Figure S3. A: Urinary levels of 8-isoprostane (A) and proteinuria (B) at baseline and 210min after PTRA in ARAS+PTRA+Vehicle and ARAS+PTRA+Bendavia. #p<0.05 vs. ARAS+PTRA+Vehicle. Neither vasoconstrictor (D) nor vasodilator (E) effect in response to Bendavia was observed in isolated renal artery rings.
Figure S4. A: Representative TUNEL (top) and caspase-3 staining in normal, ARAS, ARAS+PTRA+Vehicle, and ARAS+PTRA+Bendavia stenotic kidneys. B: Representative renal immunohistological staining with MCP-1 (top) and tubulointerstitial (CD163+) macrophages (bottom). C: Immunohistological staining with fibronectin (top) and collagen IV (bottom) in normal, ARAS, ARAS+PTRA+Vehicle, and ARAS+PTRA+Bendavia.
Figure S5. A: Renal blood flow (A, RBF), and glomerular filtration rate (B, GFR) in the contralateral kidney (CLK) of normal, ARAS, ARAS+PTRA+Vehicle, and ARAS+PTRA+Bendavia pigs. Renal function in the non-stenotic kidney was unchanged in ARAS and remained unaffected by Bendavia.
Figure S6. Representative TUNEL staining in the CLK of normal, ARAS, ARAS+PTRA+Vehicle, and ARAS+PTRA+Bendavia (top) and its quantification (bottom). *p<0.05 vs. Normal; ‡p<0.05 vs. ARAS+PTRA+Bendavia.
Figure S7. Renal production of superoxide anion in the CLK (top) and its quantification (bottom). 40X, dihydroethidium (DHE), 4',6-diamidino-2-phenylindole (DAPI). *p<0.05 vs. Normal; ‡p<0.05 vs. ARAS+PTRA+Bendavia.
Figure S8. Representative renal immunohistological staining with MCP-1 in the CLK (top) and its quantification (bottom). p<0.05 vs. Normal; ‡p<0.05 vs. ARAS+PTRA+Bendavia.
Figure S9. Representative images of tubulo-interstitial trichrome staining in the CLK of normal, ARAS, ARAS+PTRA+Vehicle, and ARAS+PTRA+Bendavia pigs (top). Quantification of tubulo-interstitial fibrosis (bottom). *p<0.05 vs. Normal; ‡p<0.05 vs. ARAS+PTRA+Bendavia.
Figure S10. Representative images of peri-glomerular trichrome staining in the CLK of normal, ARAS, ARAS+PTRA+Vehicle, and ARAS+PTRA+Bendavia pigs (top). Quantification of glomerular score (bottom). *p<0.05 vs. Normal; ‡p<0.05 vs. ARAS+PTRA+Bendavia.