Persistent kidney dysfunction in swine renal artery stenosis correlates with outer cortical microvascular remodeling


Departments of 1Internal Medicine, Division of Nephrology and Hypertension, 2Laboratory Medicine and Pathology, and 3Division of Cardiovascular Diseases, Mayo Clinic, Rochester, Minnesota

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RENA L ARTERY STENOSIS (RAS), most commonly caused by atherosclerosis, has an incidence of almost 7% in adults older than 65 years of age (21). Patients with atherosclerotic renovascular disease (ARVD) often develop renovascular hypertension; atherosclerosis; renovascular disease


Eighty-five percent of patients with renovascular hypertension have significant symptoms, yet the second most common cause of RAS, revascularization often results in improvement of both renovascular hypertension and renal hypertension; atherosclerosis; renovascular disease

METHODS

All protocols that used animals were approved by the Institutional Animal Care and Use Committee.

Thirty domestic female pigs (50–60 kg) were studied after 16 wk of observation and randomized into three groups: sham-treated pigs with early atherosclerosis + RAS (ARAS; n = 10), PTRA-treated ARAS (ARAS + PTRA; n = 10), and age- and body weight-matched controls (n = 10). At baseline, all ARAS pigs started on a high-cholesterol diet consisting of 2% cholesterol and 15% lard (TD-93296, Harlan-Teklad) to simulate the clinical situation in which diffuse early atherosclerosis precedes the stenosis. Six weeks later, animals were anesthetized with 0.5 g of intramuscular ketamine and xylazine anesthesia and then maintained with intravenous ketamine (0.2 mg·kg⁻¹·min⁻¹) and xylazine (0.03 mg·kg⁻¹·min⁻¹). RAS was induce by placing in the main renal artery a local-irritant coil, which leads to a gradual development of unilateral RAS over a 7- to 10-day period, as previously described (13). After induction of RAS, a telemetry system was implanted in the left femoral artery to continuously measure mean arterial pressure (MAP) for the 10 following wk. The average MAP in the last 2 wk of the study was subsequently calculated.

Six weeks after induction of RAS, the degree of stenosis was determined by angiography, and PTRA or sham was performed.
Briefly, a 7-mm balloon catheter wrapped with a standard tantalum stent was engaged in the proximal-middle section of the renal artery under fluoroscopic guidance, and the balloon was inflated to 8 atm, resulting in expansion of a stent to full-balloon diameter and restoration of luminal patency. Then, the balloon was deflated and removed, leaving the stent embedded in the vascular wall.

Four weeks after PTRS, the pigs were again similarly anesthetized, and angiography was repeated. Renal hemodynamics and function in each stenotic kidney were assessed using multidetector computer tomography (MDCT). Venous blood samples were collected for plasma renin activity (PRA) and creatinine measurements (Gamma-Coat PRA kit; DiaSorin, Stillwater, MN). The animals were then allowed a 3-day recovery period from the procedure and then were euthanized with a lethal intravenous dose of 100 mg/kg pentobarbital sodium (Sleepaway, Fort Dodge Laboratories, Fort Dodge, IA) (26).

The kidneys were removed using a retroperitoneal incision and immediately dissected, and sections were frozen in liquid nitrogen (and maintained at −80°C) or preserved in formalin (9). Renal fibrosis was evaluated by trichrome staining and by the expression of transforming growth factor (TGF)-β, plasminogen activator inhibitor (PAI-1), matrix metalloproteinases (MMP)-2 and MMP-9, membrane-type MMP (MT-MMP), and tissue transglutaminase (tTG). Renal hypoxia was evaluated by the expression of hypoxia-inducible factor-β1 (HIF-1β) and microvascular remodeling using micro-CT and by the expression of vascular endothelial growth factor (VEGF). Oxidative stress was assessed by the quantification of the systemic levels of oxidized low-density lipoproteins (OX-LDL) and in situ production of superoxide anion.

**In Vivo Studies**

MDCT is an ultra-fast scanner that provides accurate and noninvasive quantifications of single kidney volume, regional perfusion, renal blood flow (RBF), GFR, and tubular function (5, 8, 9, 13, 15). Briefly, 160 consecutive scans were performed following a central venous injection of iopamidol (0.5 ml·kg⁻¹·s⁻¹). Then, MDCT images were reconstructed and displayed with the Analyze software package (Biomedical Imaging Resource, Mayo Clinic, Rochester, MN). The same procedure was repeated after a 15-min interval and toward the end of a 10-min intra-aortic infusion of acetylcholine (Ach; 5 mg·kg⁻¹·min⁻¹) into a tracker catheter placed above the renal arteries to test endothelium-dependent microvascular reactivity. Hemodynamics and function were therefore measured over a stable 3-min observation period at baseline and during Ach infusion (8, 9).

For data analysis, regions of interest were selected from cross-sectional images from the aorta, renal cortex, and medulla to generate tissue attenuation curves in each region to obtain measures of renal function (15). Cortical and medullary volumes were calculated and RBF was computed as the sum of the products of cortical and medullary perfusions and corresponding volumes. GFR was assessed from the cortical curve using the slope of the proximal tubular curve.

**In Vitro Studies**

**Renal histology.** Midhilal 5-μm cross sections of each kidney (one per animal) were examined using a computer-aided image analysis program (Meta Morph, Meta Imaging, Molecular Devices, Sunnyvale, CA). In each slide, trichrome staining was semiautomatically quantified in 15–20 fields, expressed as fraction of kidney surface area, and the results from all fields were averaged. Glomerular score (% of sclerotic out of 100 glomeruli) was also assessed (8–11).

**Western blotting.** Western blotting protocols were followed using specific polyclonal antibodies against active and pro-MMP-2 and MMP-9, MT-MMP, PAI-1, TGF-β, tTG, HIF-1α, and VEGF (8, 9, 11). Protein expression was determined in each kidney, and the intensities of the protein bands (one per animal) were quantified and normalized for a GADPH loading control. MMP-2 and MMP-9 were normalized for their respective pro-MMP forms.

**Microcomputed tomography analysis.** After the kidney was flushed, microfil MV122 (an intravascular contrast agent) was perfused into the stenotic kidney under physiological pressure through a cannula ligated in a branch of the renal artery. Samples were prepared and scanned at 0.5° angular increments at 18-μm resolution, and images were analyzed as previously described (19, 47). The spatial density, average diameter, and tortuosity of microvessels (diameters 20–500 μm) in the inner, middle, and outer thirds of the renal cortex were calculated using ANALYZE and classified according to diameter as small (<40 μm), medium (40–100 mm), or large (>100 μm) microvessels (11, 47).

**Oxidative stress.** Circulating levels of OX-LDL (Mercodia, Uppsala, Sweden) were quantified from systemic venous blood samples, as we have shown before (13). In addition, in-situ renal production of superoxide anion was evaluated by fluorescence microscopy after dihydroethidium (DHE) staining (7).

**Statistical analysis.** Results are expressed as means ± SD. Statistical analysis was performed using JMP software package version 8.0 (SAS Institute, Cary, NC). Comparisons within groups were performed using paired Student’s t-test and among groups using ANOVA and unpaired t-test with Bonferroni correction. Regressions were calculated by the least-squares fit. Statistical significance for all tests was accepted for P ≤ 0.05.

**RESULTS**

All pigs had a similar body wt. Six weeks after induction of RAS, both sham- and PTRS-treated ARAS pigs achieved hemodynamically significant degrees of stenosis (37) (81.3 ± 15.7 and 76.0 ± 8.1%, respectively, P = 0.36). MAP was significantly and similarly elevated in ARAS and ARAS+PTRS (131.3 ± 32.7 and 126.1 ± 7.6 mmHg, respectively, P = 0.61) compared with normal (106.1 ± 18.1 mmHg, P = 0.02 and P = 0.05, respectively). The pigs were then randomized to PTRS or sham.

Four weeks after successful PTRS (0% stenosis in all ARAS+PTRS pigs), MAP decreased in this group (Fig. 1), but remained elevated in untreated ARAS (Table 1; P < 0.05 vs. ARAS, P = 0.43 vs. normal). Total cholesterol and LDL levels were significantly elevated in ARAS and ARAS+PTRS compared with normal (Table 1; P < 0.05 vs. normal; P > 0.05 vs. ARAS). Serum creatinine levels were significantly higher in ARAS compared with normal pigs and remained elevated after PTRS (Table 1; P < 0.05 vs. normal; P = 0.33 vs. ARAS). No significant differences in PRA were found among the groups, as typical to the chronic phase of untreated RAS (35).

**Renal Hemodynamics and Function**

Basal renal volume and GFR were significantly decreased in the stenotic ARAS compared with normal kidneys (Table 1). Cortical perfusion improved after PTRS, while RBF slightly increased (Table 1). Cortical perfusion improved after PTRS, while RBF slightly increased (Table 1). Microvessel Architecture

Transmural spatial density of cortical microvessels was significantly diminished in both ARAS and ARAS+PTRS compared with normal pigs (Fig. 2), while average vessel diameter increased (Table 2). Moreover, ARAS and ARAS+
PTRS pigs showed a similar increase in microvascular tortuosity compared with normal animals, suggesting angiogenic activity to restore microvessels (Table 2). The decrease in spatial density was maintained throughout the outer, middle, and inner cortex in both ARAS and ARAS+PTRS pigs and was particularly determined by the number of small vessels (<40 μm) that were significantly reduced in ARAS and ARAS+PTRS compared with normal (Fig. 2), suggesting a selective loss of microvessels that PTRS failed to restore.

Table 1. Systemic characteristics and single-kidney hemodynamics in normal, untreated ARAS, and ARAS pigs 4 wk after PTRS

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>ARAS</th>
<th>ARAS+PTRS</th>
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<tbody>
<tr>
<td>Number of pigs</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>48.5 ± 2.3</td>
<td>51.0 ± 8.8</td>
<td>51.1 ± 7.8</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>97.2 ± 13.0</td>
<td>114.6 ± 25.1↑</td>
<td>95.1 ± 16.0</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>124.6 ± 17.0</td>
<td>148.7 ± 31.5↑</td>
<td>125.4 ± 17.9</td>
</tr>
<tr>
<td>Mean arterial pressure, mmHg</td>
<td>106.3 ± 14.0</td>
<td>126.0 ± 26.9↑</td>
<td>105.2 ± 16.5</td>
</tr>
<tr>
<td>Degree of stenosis, %</td>
<td>0</td>
<td>92.0 ± 17.3↑</td>
<td>0</td>
</tr>
<tr>
<td>Serum creatinine, mg/dl</td>
<td>1.29 ± 0.16</td>
<td>1.79 ± 0.37*</td>
<td>1.85 ± 0.23*</td>
</tr>
<tr>
<td>PRA, ng·ml⁻¹·h⁻¹</td>
<td>0.18 ± 0.14</td>
<td>0.20 ± 0.21</td>
<td>0.27 ± 0.18</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>92.5 ± 16.0</td>
<td>511.0 ± 128.1*</td>
<td>385.7 ± 166.2*</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dl</td>
<td>47.3 ± 8.6</td>
<td>332.6 ± 127.4*</td>
<td>235.9 ± 122.3*</td>
</tr>
<tr>
<td>Oxidized-LDL, μg/ml</td>
<td>1.03 ± 0.51</td>
<td>2.76 ± 3.40*</td>
<td>1.55 ± 0.85</td>
</tr>
<tr>
<td>Renal volume, ml</td>
<td>137.1 ± 22.1</td>
<td>93.6 ± 27.6*</td>
<td>106.2 ± 21.8*</td>
</tr>
<tr>
<td>Cortical perfusion, ml·min⁻¹·ml tissue⁻¹</td>
<td>4.7 ± 1.1</td>
<td>3.7 ± 0.9*</td>
<td>4.2 ± 1.4</td>
</tr>
<tr>
<td>RBF, ml/min</td>
<td>609.9 ± 130.7</td>
<td>391.0 ± 172.2*</td>
<td>468.0 ± 222.0*</td>
</tr>
<tr>
<td>GFR, ml/min</td>
<td>77.4 ± 27.7</td>
<td>52.5 ± 14.0*</td>
<td>57.0 ± 16.2*</td>
</tr>
</tbody>
</table>

Values are means ± SD. ARAS, atherosclerosis + renal artery stenosis; PTRS, percutaneous transluminal renal stenting; PRA, plasma renin activity; RBF, renal blood flow; GFR, glomerular filtration rate; LDL, low-density lipoprotein. *P ≤ 0.05 vs. normal. †P ≤ 0.05 vs. ARAS+PTRS.
ARAS and ARAS+PTRS (but no other cortical region) correlated linearly and significantly with GFR (Fig. 2), indicating that microvascular remodeling in the outer cortex might have contributed to the residual renal dysfunction observed 4 wk after PTRS.

Oxidative Stress

Systemic OX-LDL levels were significantly higher in ARAS compared with normal, and slightly decreased after PTRS to levels that were not different from normal or sham ARAS (Table 1; $P = 0.15$ vs. ARAS+PTRS), indicating that systemic oxidative stress was improved but not abolished after revascularization. Moreover, in situ production of superoxide anion was similarly increased in ARAS and ARAS+PTRS compared with normal ($P = 0.99$ vs. ARAS; Fig. 3).

Renal Hypoxia

HIF-1 expression was significantly increased in ARAS compared with normal ($P = 0.02$) and decreased after revascularization (Fig. 3), but it was not significantly different from ARAS+PTRS ($P = 0.28$).

Renal Scarring, Fibrogenic and Angiogenic Factors

Expression of VEGF protein was reduced in ARAS ($P < 0.05$ vs. normal; Fig. 3) and further decreased after revascularization ($P < 0.05$ vs. ARAS). PTRS also failed to restore the expression of the fibrogenic factors PAI-1 ($P = 0.003$ vs. normal) and TGF-β ($P = 0.02$ vs. normal), both of which were similarly upregulated in ARAS and ARAS+PTRS. On the other hand, no significant differences were found in the levels that were not different from normal or sham ARAS (Table 1; $P = 0.15$ vs. ARAS+PTRS), indicating that systemic oxidative stress was improved but not abolished after revascularization. Moreover, in situ production of superoxide anion was similarly increased in ARAS and ARAS+PTRS compared with normal ($P = 0.99$ vs. ARAS; Fig. 3).

Table 2. Renal cortical microvascular architecture assessed by micro-CT in normal, ARAS, and ARAS+PTRS

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>ARAS</th>
<th>ARAS+PTRS</th>
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<tbody>
<tr>
<td>Transmural vessels/cm²</td>
<td>269.7</td>
<td>161.0</td>
<td>184.0</td>
</tr>
<tr>
<td>Outer cortex vessels/cm²</td>
<td>309.4</td>
<td>165.1</td>
<td>176.7</td>
</tr>
<tr>
<td>Outer cortex size-specific</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40-100µm vessels/cm²</td>
<td>286.7</td>
<td>163.0</td>
<td>190.0</td>
</tr>
<tr>
<td>40-100µm size-specific</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100µm vessels/cm²</td>
<td>212.9</td>
<td>155.0</td>
<td>185.4</td>
</tr>
<tr>
<td>100µm size-specific</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average vessel diameter, µm</td>
<td>92.02</td>
<td>105.77</td>
<td>101.98</td>
</tr>
<tr>
<td>Tortuosity ratio</td>
<td>1.32 ± 0.23</td>
<td>1.54 ± 0.39*</td>
<td>1.51 ± 0.38*</td>
</tr>
</tbody>
</table>

Values are means ± SD. *P < 0.05 vs. normal. No significant differences were found between ARAS and ARAS+PTRS pigs.
of MMP-2, MMP-9, MT-MMP, and tTG among the groups (Fig. 3).

Trichrome staining showed that tubulointerstitial fibrosis was elevated in ARAS and did not change after revascularization (P = 0.43 vs. ARAS; Fig. 4). Glomerular score (index of glomerulosclerosis) was higher in ARAS compared with normal (Fig. 4) and did not improve after revascularization (P < 0.001 vs. normal, P = 0.67 vs. ARAS). There was no significant difference in glomerular score between the outer and inner cortex in ARAS or ARAS+PTRS (P = 0.11 and P = 0.13, respectively; Fig. 4).

**DISCUSSION**

The present study demonstrates that tubulointerstitial injury and renal dysfunction in experimental ARVD persist 4 wk after PTRS, despite resolution of hypertension. Four weeks after mechanically successful revascularization, serum creatinine levels remained higher and stenotic-kidney GFR significantly reduced compared with normal pigs, and their response to Ach was blunted, suggesting that endothelial function remained impaired after revascularization. Persistent dysfunction was spatially related to outer cortical microvascular loss and accompanied by relentless oxidative stress and renal fibrosis. This study therefore supports the need for evaluation of the extent of renal parenchymal disease before revascularization in patients with ARVD.

RAS remains the major cause of renovascular hypertension and may induce renal tissue injury leading to progressive renal failure (27, 39). Atherosclerosis is responsible for as many as 90% of all cases of renovascular disease (39). Previous studies demonstrated that renal functional outcomes are dependent on intrarenal parenchymal damage in patients with ARVD (46). Moreover, revascularization improves renal function in a minority of patients with ARVD, possibly because of direct effects of diffuse atherosclerosis on the kidney (28). Indeed, we previously showed that atherosclerosis-induced oxidative stress and inflammation amplified renal dysfunction in ARAS by augmenting tubulointerstitial and glomerular fibrosis (8). We also showed that in a model of nonatherosclerotic RAS, PTRS decreased blood pressure and improved the renal microvascu-
lar network, and while tubulointerstitial injury was not fully resolved, GFR recovered 4 wk after the procedure (19). The current study extends our previous observations and demonstrates that superimposition of atherosclerosis accentuates irreversible renal injury, as it is associated with persistent renal dysfunction after successful revascularization, as observed in many patients with ARVD (20), and supports the notion that parenchymal disease diminishes response to revascularization in ARVD.

Human ARAS is often superimposed on essential hypertension or severe preexisting renal injury, which sustains systemic hypertension after PTRS. Interestingly, this study underscores the ability of PTRS to decrease blood pressure in early stages of ARAS, likely by restoration of vessel patency, increase in renal perfusion pressure, and thereby downregulation of renin-angiotensin-aldosterone system activity (which does not necessarily reflect in systemic PRA). Contrary to systemic hypertension, renal damage in our model persisted 4 wk after revascularization. Evidently, the mild residual kidney damage observed in the stenotic swine kidney is not severe enough to maintain elevated blood pressure after PTRS.

Renal damage in patients with RAS is instigated by generation of angiotensin II, which after prolonged activation stimulates deleterious pathways that lead to progressive renal injury, including inflammation, fibrogenesis, and oxidative stress. This detrimental process is commonly initiated by endothelial injury (28), which might lead to endothelial dysfunction (4), reflected in our study by blunted responses to Ach. In

Fig. 4. Top: representative images of renal trichrome staining in normal, ARAS, and ARAS+PTRS pigs (top) and its quantification (means ± SD; middle). Bottom: overall (left) and outer vs. inner (right) cortical glomerular score (% of sclerotic glomeruli). *P < 0.05 vs. normal.
addition, increased production of reactive oxygen species (ROS) in the stenotic ARAS kidney, suggested by increased renal production of superoxide anion and circulating levels of OX-LDL, might stimulate fibrogenic factors and amplify renal dysfunction and injury (8, 9). Indeed, in 2-kidney, 1-clp rats a superoxide dismutase mimetic (Tempol) improves renal oxygenation and function (33), and chronic administration of antioxidant vitamins E and C improves renal function and structure in swine RAS (11). Systemic oxidative stress was improved after revascularization, probably because of the decrease in blood pressure, but it was not abolished because the pigs remained hypercholesterolemic. However, the potential for paradoxical prooxidant effects of antioxidants (16) and disappointing results of cardiovascular clinical trials do not support routine use of antioxidants in patients with ARVD.

Persistent renal fibrosis was likely secondary to upregulation of the fibrogenic factors TGF-β and PAI-1 in the atherosclerotic milieu. These may be induced by angiotensin II, ROS, cytokines, and growth factors (17) and might have accounted for the continued glomerulosclerosis and tubulointerstitial fibrosis after PTRS. While chronic reduction of renal perfusion in ARAS may modulate microvascular structure (remodeling or regression) (1, 22, 42), in our model these were likely not mediated by the vascular remodeling factors (TG or MPP, because their levels were not different than normal.

Although PTRS restored the increased levels of HIF-1α, expression of the downstream angiogenic factor VEGF was decreased in ARAS and ARAS + PTRS, possibly mediated by oxidative stress, which may impair the ability of the kidney to generate new vessels. We observed decreased spatial density and selective loss of small microvessels in the renal cortex, and thereby an increase in average diameter of the remaining vessels. Considering the relatively mild glomerulosclerosis, the lost microvessels were likely primarily nutrient. Interlobular arteries may branch into a plexus of nutrient capillaries or perforating capular arteries, which do not necessarily pass through the glomerulus (31). Furthermore, in the pig kidney afferent arterioles often give off small (<40 μm) secondary and tertiary branches (43). The increased microvascular tortuosity in ARAS suggests some formation of new vessels, yet this compensatory mechanism was not fully effective, possibly because of downregulation of VEGF due to high levels of ROS (23).

Previous studies documented the effect of loss (rarefaction) or damage of renal microvessels on progression of renal injury (25), deterioration of renal function, and glomerular and tubulointerstitial scarring (9, 12). Interestingly, the spatial density of microvessels located specifically in the outer cortex correlated linearly with GFR, underscoring the contribution of microvascular remodeling in this region to renal dysfunction in ARAS. This spatial selectivity might be partly related to anatomical and functional differences between the outer and deep renal cortex. Approximately 70% of RBF is distributed to the outer half of the cortex (40), which is selectively affected by a decrease in renal perfusion pressure (29, 30). Speculatively, microvascular loss in this region may be vital for renal function, as supported by the relative abundance of glomeruli in the outer third of the swine renal cortex (43). Furthermore, efferent arterioles located in the outer and middle cortex tend to perfuse nephron segments located in various cortical regions (3, 18), and might thus be more critical determinants of GFR than inner cortical efferent arterioles.

Limitations

Our study is limited by the use of relatively young animals and short duration of the disease. Alas, renal structure and function in our swine model are similar to human kidneys, and superimposition of atherosclerosis in RAS also accelerates renal functional compromise. We also observed that PTRS decreased blood pressure, which is not a consistent observation in ARVD. Indeed, human ARVD is multifactorial and prolonged, and often associated with other concurrent or preexisting pathophysiologic conditions like essential hypertension, which account for unaltered blood pressure levels after revascularization. Our study therefore highlights the dissociation of potential benefits of revascularization for blood pressure reduction from its efficacy for renal function improvement in ARAS. Furthermore, a longer observation period may be needed to detect measurable improvement in renal function and structure.

In summary, the current study shows that tubulointerstitial injury, microvascular rarefaction, and renal dysfunction persisted after revascularization in swine ARAS. Moreover, we found that microvascular loss in the outer cortex might be an important determinant of persistent renal dysfunction after PTRS. Therefore, our study suggests a central role for outer cortical microvessels in maintaining GFR in ARVD and supports exploration of vasculo-protective strategies, such as administration of VEGF (6) or progenitor cells (12), for preserving microvascular integrity and function in the stenotic ARAS kidney. Finally, this study underscores the need for assessment of parenchymal disease in the selection of patients for revascularization.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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