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Disparate effects of single endothelin A and B receptor blocker therapy on the progression of renal injury in advanced renovascular disease

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Abstract

We hypothesized that chronic specific endothelin (ET)-A receptor blockade therapy would reverse renal dysfunction and injury in advanced experimental renovascular disease. To test this, unilateral renovascular disease was induced in 19 pigs and after 6 weeks, single-kidney hemodynamics and function was quantified *in vivo* using computed-tomography. All pigs with renovascular disease were divided such that 7 were untreated, 7 were treated with ET-A blockers, and 5 were treated with ET-B blockers. Four weeks later, all pigs were re-studied *in vivo*, then euthanized and *ex vivo* studies performed on the stenotic kidney to quantify microvascular density, remodeling, renal oxidative stress, inflammation, and fibrosis. RBF, GFR, and redox status were significantly improved in the stenotic kidney after ET-A but not ET-B blockade. Furthermore, only ET-A blockade therapy reversed renal microvascular rarefaction and diminished remodeling, which was accompanied by a marked decreased in renal inflammatory and fibrogenic activity. Thus, ET-A but not ET-B blockade ameliorated renal injury in pigs with advanced renovascular disease by stimulating microvascular proliferation and decreasing the progression of microvascular remodeling, renal inflammation and fibrosis in the stenotic kidney. These effects were functionally consequential since ET-A blockade improved single kidney microvascular endothelial function, RBF, and GFR, and decreased albuminuria.

Keywords

kidney; hemodynamics; vascular regulation; renal artery stenosis; endothelin; microcirculation; imaging

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Introduction

Renal artery stenosis is a major cause of chronic renovascular disease (RVD) in the US adult population that could eventuate in chronic kidney disease and end stage renal disease[1]. Chronic RVD has a higher prevalence in older population, affecting almost 18% of individuals between the ages 65 to 74 years and over 40% of those older than 75[2], significantly increasing health care costs.

Endogenous endothelin (ET) is a complex autocrine and paracrine system that has specific actions in the kidney and contributes to regulation of blood pressure and renal function. ET-1, the major isoform of the ET family, exerts its effects through the specific ET-A and ET-B receptors. In general, ET-A receptors mediate vasoconstriction, nerve stimulation, and cell proliferation, whereas ET-B receptors are involved in ET-1 clearance, release of nitric oxide (NO), and inhibition of the ET converting-enzyme. In the kidney, ET receptors are found throughout the vascular, tubular, and glomerular compartments, and ET-1 is regulated in an autocrine/paracrine manner showing a prolonged and long-lasting binding especially to the ET-A receptors. Activation of ET-A receptors promotes renal vasoconstriction and cell proliferation. In contrast, the ET-B receptor promotes sodium and water excretion and minimizes the binding of ET-1 to ETA, thereby opposing renal vasoconstriction[3].

While ET helps to maintain normal renal function and blood pressure, over-activation of the renal ET system may contribute to the initiation and progression of chronic kidney disease in diabetes, hypertension, and glomerulonephritis. ET-1 has been shown to promote renal inflammation and fibrosis via ET-A receptors whereas chronic ET antagonism can mitigate the decline in renal function and renal damage in acute renal ischemia and atherosclerosis. We have recently shown in a proof-of-concept study (using a fully characterized, clinically relevant swine model of experimental RVD[4–6]) that chronic ET-A blockade *from the onset of RVD* preserves the function and microvascular density of the stenotic kidney, and attenuated renal fibrosis, implicating a role of the ET-1/ET-A pathway on the development of renal injury[7]. On the other hand, these results also opened the possibility that a large portion of the beneficial effects of ET-A blockade in the kidney may be due to increased availability of ET-1 to bind the ET-B receptors, and thus stimulating vasodilatation, opposing microvascular rarefaction, and decreasing renal injury. However, little is known about the role of ET-B receptors in preserving (or not) microvascular structure and function in the stenotic kidney. Furthermore, whether specific ET receptor blockade could reverse renal injury in established RVD has not been yet determined. Thus, the current study was designed to test the hypothesis that chronic specific blockade of the ET-A receptors will reverse or slow the progression of renal damage in the stenotic kidney (in advanced RVD) largely by protecting the intra-renal microvascular architecture and function. Furthermore, this study will determine, for the first time, the relative contributions of ET-A and ET-B receptors to the progression of renal injury in chronic RVD. These studies could lead us to the identification of potential therapeutic targets and novel interventions to slow the progression of renal injury in RVD.

Results

ET in RVD

Plasma levels of ET-1 measured from renal venous blood of the stenotic kidney were elevated in RVD compared to normal pigs (0.59±0.03 and 0.23±0.01 pg/mL, respectively, $p<0.05$ vs. Normal), not modified by ET-A blockade (0.64±0.06 pg/mL, $p<0.05$ vs. Normal, p=NS vs. RVD), but further elevated after ET-B blockade (0.98±0.04 pg/mL, $p<0.05$ vs. Normal, RVD, and RVD+ET-A), suggesting that blockade of the ET-B receptor was effective and supporting the role of the B receptors in the clearance of ET-1.

General characteristics

Body weight was similar in all animals after 6 and 10 weeks of observation (Table 1 and 2). The angiographic degree of stenosis was similarly and significantly greater in all RVD pigs and not modified by ET-blockers (Table 1 and 2). Hypertension was similar in all pigs with RVD at 6 weeks (Table 1). However, 4 weeks of ET-A blockade induced a slight but not significant attenuation of hypertension compared to 6-weeks pre-treatment values that resulted in a significant difference compared to untreated RVD at 10 weeks (Table 2). Plasma renin activity (PRA) was similar among the groups at 6 and 10 weeks (Table 1 and 2), as we have previously shown[8] and has been observed in the chronic phase of renovascular hypertension[9,10]. Serum creatinine was similarly and significantly elevated in all RVD pigs at 6 weeks compared to normal, but showed a further increase of 25% at 10 weeks ($p<0.05$ compared to 6 weeks) in untreated RVD whereas remained virtually unchanged (−2.5%, p=NS compared to 6 weeks) in ET-A blocker-treated pigs (Table 1 and 2). Finally, the increased albuminuria at 6 and 10 weeks in untreated RVD was substantially reduced after 4 weeks of ET-A blocker therapy (Figure 1).

Multi-detector computed tomography (MDCT)-derived single-kidney function

Basal and acetylcholine (Ach)-stimulated renal blood flow (RBF) and glomerular filtration rate (GFR) were reduced in the stenotic kidney of RVD (at 6 and 10 weeks), but substantially augmented after 4 weeks of treatment with ET-A blockers (Table 1 and 2, Figures 1 and 2), accompanied by a significant decrease in renal vascular resistance, decreased uncoupling of endothelial NO synthase (eNOS, Figure 2), decreased superoxide production (Figure 2), and augmented superoxide dismutase (SOD) activity in RVD+ET-A ($p<0.01$ vs. RVD, p=NS vs. Normal, Table 2).

Microvascular 3D architecture

The density and distribution of microvessels of diameter under 500 μm in the stenotic RVD kidney (which includes interlobar, arcuate, radial, and smaller branching orders microvessels[11]) were significantly decreased throughout the renal cortex and medulla, accompanied by increased media-to-lumen ratio ($p<0.05$ vs. Normal), suggesting marked microvascular rarefaction and remodeling in the stenotic kidney. Notably, cortical and medullary microvascular density was significantly augmented in RVD+ET-A treated kidneys (p=0.05 vs. RVD, Figure 3-top) and was not different than in normal kidneys (p=0.24 vs. Normal) on those microvessels under 200μm in diameter whereas microvessels

between 200–500μm in diameter remained attenuated (Figure 3). However, renal expression of tissue-transglutaminase (tTg) and plasminogen (Plg) (Figure 4), and microvascular media-to-lumen ratio (Figure 5) were significantly reduced compared to RVD, which suggests a vascular protective effect of ET-A blockers in the microvasculature of the stenotic kidney.

Angiogenic factors

The stenotic kidney showed a significant decrease in the expression of pro-angiogenic vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and of downstream mediators such as phosphorylated (p)-Akt and angiopoietin (Ang)-1, whereas angiostatin and endostatin were elevated. Notably, chronic ET-A blockade in RVD significantly augmented the expression of VEGF, HGF, p-Akt, and Ang-1, and normalized angiostatin and endostatin (ANOVA $p<0.05$ for all), indicating a pro-angiogenic and pro-survival effect that associates with the improved cortical and medullary microvascular density in the RVD+ET-A kidney (Figure 4a).

Renal markers of inflammation and fibrosis

The renal expression of pro-inflammatory nuclear factor kappa (NFk)B, tumoral necrosis factor (TNF)-α, and monocyte chemoattractant protein (MCP)-1 was significantly increased in the stenotic kidney compared to normal controls. All these factors were substantially reduced after ET-A blockade (Figure 4b, ANOVA $p<0.05$ for NFkB), implying attenuated pro-inflammatory activity. On the other hand, the expression of pro-fibrotic transforming growth factor (TGF)-β that was significantly increased in RVD was reduced by ET-A blockade (ANOVA $p<0.05$), accompanied by augmented renal expression of matrix-metalloproteinase (MMP)-2 (ANOVA $p<0.05$), p-proliferating cell nuclear antigen (p-PCNA) and decreased expression of caspase-3 and -8, suggesting improved renal turnover of extracellular matrix, remodeling and attenuated cell damage (Figure 4b).

Renal morphology

The degree of glomerulosclerosis (Figure 5) and the fraction of apoptotic cells were elevated in RVD compared to normal kidneys (16.2±0.7 and 1.8±0.2%, respectively, $p<0.05$ vs. Normal). Notably, administration of ET-A blockers significantly reduced glomerulosclerosis (Figure 5) and the fraction of apoptotic cells (7.02±0.1%, $p<0.05$ vs. Normal and RVD).

Effects of ET-B blockers on renal function and microvascular architecture

Serum creatinine increased (albeit not significantly) 15% after 4 weeks of ET-B blockade (Table 3). Furthermore, ET-B receptor blocker therapy did not significantly improve basal (compared to 6 weeks, Table 3) or stimulated RBF or GFR values at 10 weeks (3% and 12%, respectively, p=NS vs. baseline), indicating a persistence of microvascular endothelial dysfunction. These were accompanied by blunted renal SOD activity ($p<0.05$ vs. Normal and RVD+ET-A, p=NS vs. RVD) and a persistent increase of eNOS uncoupling and superoxide anion in the stenotic kidney (Figure 6), suggesting increased oxidative stress and a potential diminished renal bioavailability of NO. Furthermore, chronic treatment with ET-B blockers did not recover microvascular rarefaction and further increased media-to-lumen

ratio, indicating persistence of microvascular damage and limited effects on microvascular proliferation and repair in the stenotic kidney (Figure 6). Glomerulosclerosis in the stenotic kidney remained similarly elevated compared to untreated RVD (Figure 6).

Discussion

The current study extends previous observations by we[7,12,13] and others[14] and demonstrates that ET-A blockade therapy is capable of halting or even reversing the progression of renal injury in advanced RVD and established renal damage. Furthermore, using a model of progressive renal injury, this study helps to determine the relative contributions of the ET receptors for the progression of renal dysfunction and damage (or mediate renoprotection) in the stenotic kidney. We also show that blockade of the ET-B receptors does not result in an effective therapeutic intervention for the stenotic kidney in RVD as observed after blockade of the ET-A receptors. Indeed, our data show that ET-A blockade exerts vasculo- and reno-protective effects on the stenotic kidney, by decreasing the progression of microvascular remodeling, inflammation and fibrosis, and by stimulating microvascular proliferation. Importantly, these effects were functionally consequential since ET-A blockade therapy for 4 weeks also improved renal microvascular endothelial function, consequently recovering stenotic kidney RBF and GFR and diminishing hypertension.

Chronic RVD (mainly due to renal artery stenosis) is observed in up to 60% of patients with overt coronary artery disease, aorto-iliac disease, or peripheral vascular disease[15]. RVD is an independent predictor of cardiovascular mortality and a cause for a progressive deterioration of renal function, which may lead to chronic renal disease and end-stage renal disease[16,17]. A sustained obstruction of blood flow into the kidney and deficient perfusion pressure serves as a potent stimulus that leads to renal dysfunction and structural injury, which progresses as RVD evolves.

ET-1 is the major isoform of the ET pathway that is ubiquitously produced and expressed in the kidney throughout the vascular, glomerular, and tubular compartments. The kidney is a source and a target for ET-1, which is a powerful vasoconstrictor and mitogenic peptide that exerts its effects through two specific G-protein coupled receptors, ET-A and ET-B[18]. An elevated renal production of ET-1 has been suggested as both a marker and a promoter of renal damage[7,19] and a recent study supports a role for ET-1 (through ET-A receptors) in initiating CKD/ESRD after experimental ischemia-reperfusion injury[14]. The specific actions of ET have provided the rationale for selective therapeutic blockade of ET-A receptor in pathological settings such as acute renal ischemia[14,20,21], hypertension[3,22], and atherosclerosis[12,13]. These studies also suggest that leaving the ET-B receptors unmodified may be an important part of this therapeutic approach due to their beneficial actions upon activation. However, specific pathological conditions such as pulmonary hypertension are ameliorated when blockade of both ET-A and ET-B receptors is achieved[3], suggesting that the actions mediated by each receptor might differ depending on the tissue, organ, or, possibly, the disease. Although ET-A and -B receptors have opposing roles in vascular tone and cell proliferation, it seems that in certain pathological situations they can elicit similar actions[23]. Therefore, despite the evidence supporting the targeting of the ET-A receptors for

therapeutic interventions in RVD[7], it is unknown whether blockade of the ET-B receptors in RVD may induce renoprotection.

Our study shows that chronic specific blockade of the ET-A receptor led to a significant recovery of the renal hemodynamics and function of the stenotic kidney, which was observed not only at baseline but also after endothelial-dependent vasodilatation. These changes were accompanied by augmented microvascular density in both cortex and medulla, indicating that ET-A blockade may have efficiently protected microvascular endothelial function and integrity in the stenotic kidney. We demonstrated that there is a marked microvascular rarefaction in the stenotic kidney after 6 weeks of experimental RVD and that such changes could be largely prevented by ET-A blockade when administered from the onset of the disease[4]. We are now extending those observations by reporting that 4 weeks of ET-A blocker therapy in advanced RVD and established renal damage may reverse the progression of renal injury. The improvement of the microvascular architecture after ET-A blocker therapy in the current study was evident on the smaller microvessels of both cortex and medulla, indicating a possible sprouting of small vessels from pre-existing ones. It is likely that the large recovery of renal function we observed in the current study was a combined therapeutic effect on the microvascular structure and function. Blockade of the ET-A receptors may have preserved endothelial cells from ischemia-induced damage[20]. These effects were accompanied by a milder hypertension and a significant decreased albuminuria in ET-A blocker treated animals compared to untreated RVD, which possibly reflect glomeruloprotective[24,25] effects of ET-A blockade therapy on the stenotic (and possibly contralateral) kidney. In addition, previous studies have also shown distinct blood pressure-independent effects of ET-A blockade on reducing proteinuria[26–28]. The latter might have served as an additional protective mechanism of ET-A blocker therapy, although we cannot definitively conclude this from our experimental design.

Chronic blockade of the ET-A receptors also reduced the microvascular media-to-lumen ratio and the expression of tTg, markers of microvascular remodeling[29]. Furthermore, it reduced the expression of Plg, suggesting a reduction in microvascular permeability and damage[11], which likely extends to new and pre-existing renal vessels. It is also possible that the large recovery of renal function after ET-A blockade was partly due to a powerful hemodynamic effect on new and likely on pre-existing (repaired or intact) vessels (also possibly via ET-1/ET-B receptor-mediated actions[18]). The improvements in renal hemodynamics may have been the combined result of a reduction of uncoupled eNOS, decrease in renal superoxide, and improved scavenging activity of SOD. We have shown that the stenotic kidney has a significant oxidative stress[29–31] and that ET-A blockade exerts powerful renal anti-oxidant effects[13]. A reduction in superoxide abundance after ET-A blockade may have reduced quenching of NO[32], which may have increased the bioavailability of tetrahydrobiopterine (BH4) and hence reduced eNOS uncoupling[33] in the stenotic kidney. The reduction in uncoupling of eNOS after ET-A blockade may have favored renal production of NO as in turn blunted an additional source of superoxide anion[34], overall contributing to increase the biovailability of NO in the stenotic kidney. Consequently, we observed a marked improvement in basal and stimulated RBF and GFR in RVD+ET-A treated pigs.

The increased eNOS-derived NO may have in turn played a role in augmenting and mediating the effects of the increased renal VEGF after ET-A blockade, a major contributor for maintaining microvascular homeostasis[35,36]. ET-A blockade also augmented renal expression of HGF, a pleiotropic growth factor with robust angiogenic effects, both directly and via interactions with VEGF[37], with shared downstream mediators and possibly additive or synergistic effects on microvascular proliferation and repair[38,39]. Among these shared mediators are p-Akt and eNOS, which were augmented after ET-A blockade and play important roles in endothelial cell survival, microvascular proliferation, and microvascular function[40–42]. In addition, ET-A blockade therapy may have favored microvascular proliferation by also blunting the expression of powerful anti-angiogenic factors such as endostatin and angiostatin[43,44] in the stenotic kidney.

In addition, ET-A blockade resulted in a substantial reduction in the expression of NFkB, which is a pivotal promoter of inflammation. We have shown that ET-1 up-regulates renal NFkB in early atherosclerosis mainly via its ET-A receptors[12], and this study confirms and extends those observations to RVD. This factor also participates in apoptosis, a deleterious mechanism that may precede and promote renal fibrosis[45], which is increased in the stenotic kidney[5,46]. The up-regulation of NFkB was accompanied by increased expression of caspase-3 and -8, which are key players in the apoptotic cascade[46,47]. However, it has been shown that NFkB may also serve as a pro-survival factor depending on the insult[48]. Thus, the up-regulation of this factor might have been part of a compensatory mechanism to counteract the caspase-triggered pro-apoptotic activity in the stenotic kidney. Apoptosis was mainly evident at the peritubular compartment, and accompanied by a significant cortical microvascular loss, inflammation, and tubulo-interstitial, glomerular, and perivascular fibrosis[4,5,8]. Previous studies showed that ET can promote apoptosis through the ET-A receptor and could be prevented or reversed by ET antagonism[28]. The current study extends those observations and shows that the marked increase in apoptotic activity in the chronic RVD kidney was reduced by specific blockade of the ET-A receptors and accompanied by increased expression of PCNA, suggestive of augmented cell repair[49]. Since previous studies have shown anti-apoptotic effects of VEGF[50] and HGF[51], it is possible that the dual increase in VEGF and HGF in RVD+ET-A may have an additive protective effect on renal endothelial cells by augmenting the expression of pro-survival factors such as Akt[52] and attenuating caspase-3 and -8[53], thus diminishing renal apoptosis and microvascular damage.

Chronic blockade of the ET-A receptor in advanced RVD was also capable of reducing renal fibrosis, as shown by the attenuation in glomerulosclerosis and tubulointerstitial fibrosis, mainly at the renal cortical level. The reduction in renal fibrosis was associated and likely mediated by the augmented renal expression of HGF, which is also a powerful anti-fibrotic factor that counteracts the pro-fibrotic actions of TGF-β[54]. Indeed, renal expression of TGF-β (although not smad-4) was also decreased after ET-A blockade, accompanied by augmented expression of MMP-2, indicating improved turnover of extra-cellular matrix and decreased renal remodeling. In turn, augmented MMP-2 (together with VEGF) may have also contributed to the expansion of the renal microvascular networks after ET-A blockade[55–57].

The supporting evidence of the beneficial effects of ET-A receptor blockade on the function, structure, and microvascular integrity of the stenotic kidney offer a promising potential for therapeutic use in established RVD. The studies using ET-B blockers in RVD expanded these observations by suggesting that it may be important to have the ET-B receptors unmodified in this disease. The role of ET-B receptors in mediating vasodilatation and contributing to renal function was unraveled by the lack of improvements in RBF and GFR, and persistence of increased oxidative stress and potential decreases in the renal bioavailability of NO after 4 weeks of ET-B blockade. This parallel study also suggests that the ET-B receptors are possibly necessary to protect or recover the renal microvascular architecture, as implied by the persistence of microvascular rarefaction and increased remodeling. It is intriguing that cortical microvascular density showed a slight increased compared to untreated RVD (although remained significantly decreased compared to normal controls). ET-B blockers increased the renal expression of VEGF in the stenotic kidney (not shown), which was not accompanied by augmented eNOS or Akt. Speculatively, ET-B blockade may have favored pathological proliferation of renal microvessels, which were likely non- or dysfunctional (as shown by the lack of contributions to renal function) and may contribute to accentuate the progression of renal damage as RVD evolves. However, the mechanisms behind this small increase in microvascular density are not completely clear and future studies in other experimental platforms (e.g.: in vitro studies, KO models) will be needed to further understand the definitive role of ET-B receptors on renal microvascular proliferation, function, and damage in chronic RVD. On the other hand, future studies *stimulating* the ET-B receptors may offer more insights about mechanisms and applicability of this strategy, which may lead into development of a novel therapeutic alternative. Finally, since blocking one receptor may influence the activity of the other receptor [58,59], future studies using a combined ET-A and -B receptor blockade strategy in RVD will further define their roles in the disease and their potential as therapeutic targets.

In summary, our results represent a solid step in defining the role of ET-1/ET-A interactions in promoting renal injury in chronic RVD. In turn, they imply that renal function should be carefully followed when ET-B blockade is part of the treatment, as combined ET-A/B receptor blockade is currently approved for pulmonary hypertension. The current studies extend our previous observations by showing the potential of ET-A blockade as a therapeutic strategy to protect the stenotic kidney in advanced RVD with established renal damage. We are aware that our therapeutic interventions were performed at a relatively early stage of the disease. However, we believe that these studies may open new avenues for future research to elucidate the potential of ET-A blockade in reversing renal injury at more advanced stages of RVD, with different degrees of severity and concomitant insults (e.g.: lipid abnormalities, diabetes), which could better reproduce the clinical scenario in RVD and shorten the passage from pre-clinical studies towards patients in the near future.

Methods

The Institutional Animal Care and Use Committee at the University of Mississippi Medical Center approved all the procedures. Twenty-six pre-juvenile domestic pigs (sus scrofa domestica) were used for the study, which were observed for a total of 10 weeks. In 19 pigs, unilateral renal artery stenosis was induced at baseline by placing a local-irritant copper coil

inside the main renal artery. This intervention induces a progressive narrowing of the renal artery, resulting in a hemodynamically significant stenosis and constituting a surrogate of RVD, as previously shown[4,8]. Consequently, the pigs developed hypertension and a progressive renal dysfunction. Blood pressure was continuously measured by telemetry (PhysioTel, Data Sciences International) and recorded at 5-minute intervals and averaged for each 24-hour period, as described [4,8,60]. Additional pigs were used as normal controls (normal, n=7).

Six weeks after induction of RVD, all pigs were anesthetized with intra-muscular telazol (5 mg/kg) and xylazine (2 mg/kg), intubated, and mechanically ventilated on room air. Anesthesia was maintained with a mixture of ketamine (0.2 mg/kg/min) and xylazine (0.03 mg/kg/min) in normal saline, and administered via an ear vein cannula (0.05 mL/kg/min). Pigs then underwent renal angiography to quantify the degree of renal artery stenosis, as described[5,37,48]. After angiography, the catheter was positioned in the superior vena cava, and *in vivo* helical multi-detector computer tomography (MDCT) flow studies were performed for quantification of single-kidney RBF, perfusion, and GFR at baseline and after endothelium-dependent challenge via intra-renal infusion of Ach, as described[4].

Immediately after completion of the 6 week MDCT studies, the RVD pigs were then randomized (double blind, placebo-controlled) into 4 groups: placebo (RVD, n=7), those chronically treated with a specific ET-A receptor blocker (ABT 627, 0.75 mg/kg/day PO, RVD+ET-A, n=7), or a smaller group treated with specific ET-B receptor blocker (A-192621, 1 mg/kg/day PO, RVD+ET-B, n=5). Treatment or placebo was maintained for 4 additional weeks, blood pressure continuously monitored by telemetry, and at 10 weeks, MDCT in vivo studies were repeated as described for 6 weeks.

Renal vascular resistance was calculated at 6 and 10 weeks, as recently described[61]. Blood from the inferior vena cava and renal veins (from the stenotic kidney) and urine were collected at 6 and 10 weeks to measure plasma renin activity (PRA), serum creatinine (SCr), and circulating ET-1 and protein in urine by ELISA following vendors' instructions.

Upon completion of all the 10 weeks-*in vivo* studies, the pigs were allowed 48–72 hours to recover and then euthanized by an intravenous overdose of sodium pentobarbital (100mg/kg). Kidneys were immediately removed using a retroperitoneal incision and immersed in heparinized saline (10 units/mL). One lobe was used for micro-CT reconstruction. Another lobe was removed and snap-frozen in liquid nitrogen and stored at −80 ºC to quantify the protein expression of mediators of renal inflammation, fibrosis, apoptosis, and vascular proliferation and repair (Please see below). Renal tissue was also used to quantify the activity of superoxide dismutase, a potent scavenger of reactive oxygen species, by ELISA (Superoxide Dismutase Activity Colorimetric Assay Kit, Abcam, Cambridge, MA), whereas superoxide production in the stenotic kidney was measured by lucigenin luminescence, as shown[46,62]. Finally, a portion of the kidney was preserved in 10% formalin and used to investigate renal morphology in mid-hilar renal cross-sections stained with trichrome and H&E.

MDCT analysis

Manually-traced regions of interest were selected in MDCT images in the aorta, renal cortex, medulla, and papilla, their densities were sampled and time-density curves generated. The area under each segment of the curve and its first moment were calculated using curve-fitting parameters and used to calculate single-kidney RBF (ml/min), GFR (mL/min), and renal perfusion (ml/minute/cc tissue), using previously-validated methods[4,63,64].

Micro-CT

The stenotic kidney was perfused (Syringe Infusion Pump 22, Harvard Apparatus, Holliston, MA) with an intravascular contrast agent (Microfil MV122, Flow Tech, Inc., Carver, MA), samples scanned at 0.3° increments using a micro-CT scanner and reconstructed at 9 μm resolution for subsequent analysis, as described[8]. Images were analyzed with the Analyze® (Biomedical Imaging Resource, Mayo Clinic, Rochester, MN). The cortex and medulla were tomographically divided and the spatial density and distribution of microvessels (diameters <500μm) calculated as described[8,65].

Western blotting

Standard blotting protocols in renal cortical tissue homogenates were followed, as previously described[5], using specific polyclonal antibodies against mediators of angiogenesis such as VEGF, HGF, Ang-1, endostatin and angiostatin. Renal expression of factors involved in microvascular remodeling and permeability such as Plg and tTg were also determined. Furthermore, renal expression of pro-inflammatory mediators NFkB, TNF-α, and MCP-1, pro-fibrotic TGF-β, its specific mediators smad-4, pro-cell survival p-PCNA and total and p-Akt, and pro-apoptotic caspases 3 and 8 were also measured to investigate renal inflammation, fibrosis, and apoptosis, respectively. β-actin (Sigma, Saint Louis, MO, 1:500) was used as loading control. Protein expression (one band per animal) was quantified using densitometry and averaged in each group.

In addition, low-temperature SDS-PAGE was performed for the detection of eNOS dimers in renal cortical tissue homogenates as previously described[66]. Total protein was incubated at 37 degree C for 5 mins with 1x Laemmli buffer without 2-mercaptoethanol. Gels and buffers were equilibrated at 4 degree C before samples underwent SDS-PAGE with 6% gel. Running buffer tank was placed in an ice bath during electrophoresis to maintain low temperature of gel <15 degree C. After low-temperature SDS-PAGE, gels were transferred and gauged as routine western blot. Expression was quantified and expressed as the monomer/dimer ratio, as described[66].

Histology

Mid-hilar 5 μm cross sections of each kidney (1 per animal) were examined. In each slide, trichrome staining was semi-automatically quantified in 15–20 fields using a computer-aided image-analysis program (NIS Element 3.0, Nikon Instruments, Melville, NY), expressed as percentage of staining of total surface area, and the results from all fields averaged. Glomerular score was assessed by recording the number of sclerotic glomeruli out of 100 counted glomeruli, as described[4,8]. In order to quantify apoptosis, the fraction of

apoptotic cells was calculated in 10 randomly selected fields in each slide (one per animal), as described[46].

Statistical Analysis

Results are expressed as mean ± SEM. Comparisons within groups were performed using paired student's t-test, and among groups using one-way ANOVA, with Bonferroni correction for multiple comparisons. Statistical significance was accepted for $p \leq 0.05$.

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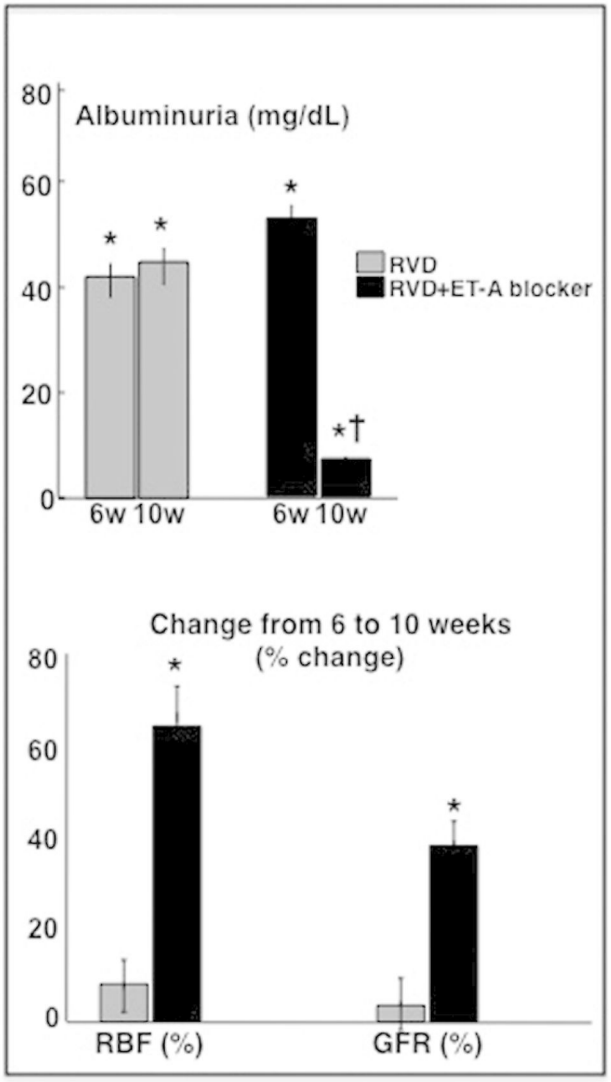


Figure 1.

Representative bar graph showing quantification of albuminuria (top) and the improvements in RBF and GFR of the stenotic kidney (bottom, % change) of animals with renovascular disease (RVD) and RVD treated with ET-A blockers for 4 weeks. ET-A blocker therapy in RVD improved RBF, GFR and significantly reduced albuminuria, suggesting reduction in microvascular damage. *p<0.05 vs. Normal, †p<0.05 vs. RVD, # p<0.05 vs. 6 weeks.

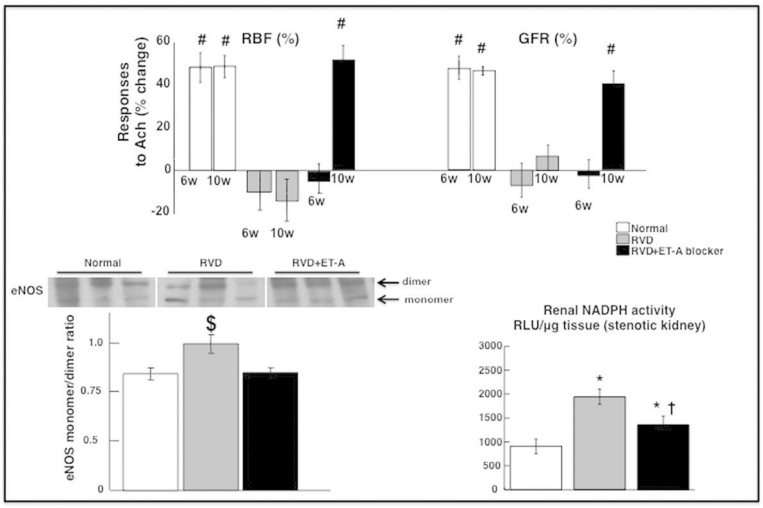


Figure 2.

Top: Representative bar graph showing the responses (% change) of RBF and GFR to an intra-renal infusion of the prototypical endothelial-dependent vasodilator acetylcholine (Ach). **Bottom-left**: Representative renal protein expression of eNOS (n=7 per group, 3 representative bands per animal shown) and quantification (expressed as ratio) from samples subjected to low-temperature (LT) SDS-PAGE to assess eNOS dimers and monomers (suggestive of eNOS uncoupling). **Bottom-right:** Quantification of NADPH-stimulated renal superoxide production in the stenotic kidney of normal, renovascular disease (RVD) and RVD treated with ET-A blockers for 4 weeks. Treatment with ET-A blockers significantly improved the expression of uncoupled eNOS, decreased renal production of superoxide, and restored microvascular endothelial function. #p<0.05 vs. Baseline; $ p=0.07 vs. Normal.

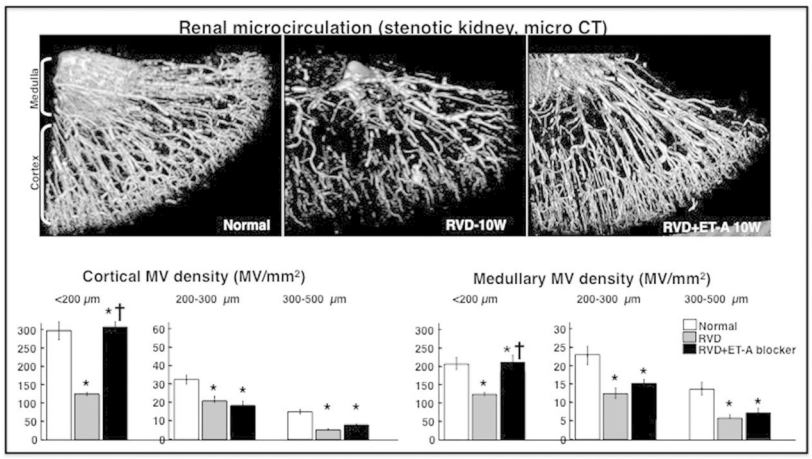
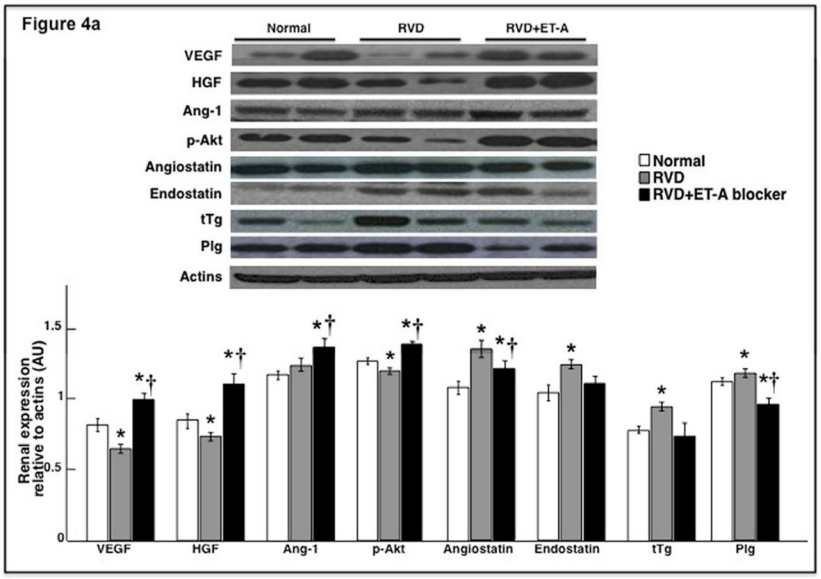


Figure 3.

Representative 3D micro-CT reconstruction (top-right) and quantification (bottom) of the cortical and medullary microvascular density (diameters under 500 μM) of the stenotic kidney after 4 weeks of ET-A blocker therapy. Chronic blockade of the ET-A receptor increased cortical and medullary microvascular density in the stenotic kidney, mainly of the smaller microvessels. *$p<0.05$ vs. Normal, $^{\dagger}p<0.05$ vs. RVD.



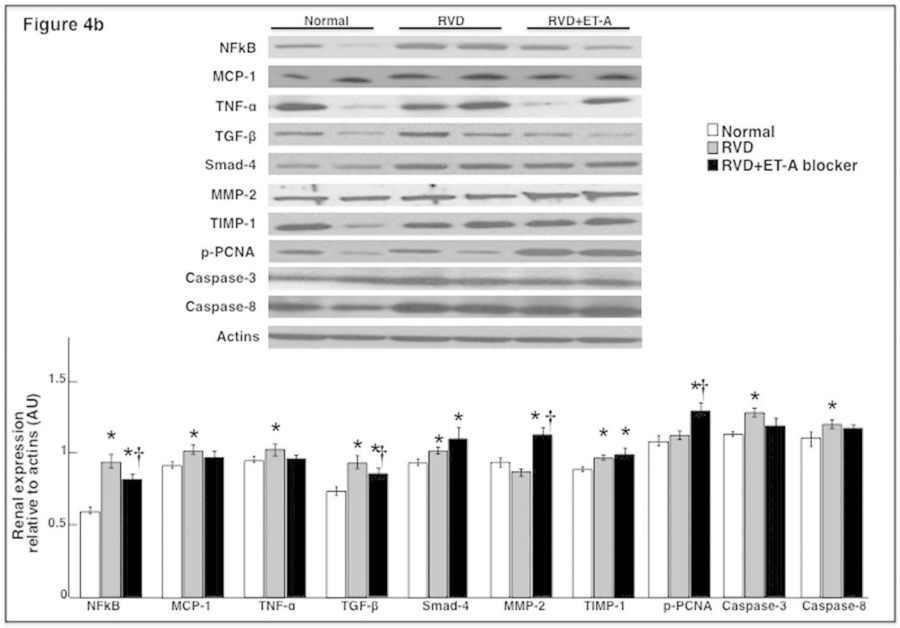


Figure 4.

a) Representative renal protein expression (n=7 per group, 2 representative bands per animal shown) and quantification of vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), angiopoietin (Ang)-1, phosphorylated (p)-Akt (normalized to total Akt), angiostatin, endostatin, tissue-transglutaminase (tTg) and plasminogen (Plg) in normal kidneys and in the stenotic kidney of pigs with renovascular disease (RVD) and RVD treated with ET-A blockers for 4 weeks. **b)** Representative renal protein expression (n=7 per group, 2 representative bands per animal shown) and quantification of nuclear factor kappa (NFk) B, monocyte chemoattractant protein (MCP)-1, tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β and smad-4, matrixmetalloproteinase (MMP)-2 and

tissue-inhibitor of MMP (TIMP)-1, phosphorylated (p)-PCNA, and caspase-3 and -8 in normal kidneys and in the stenotic kidney of pigs with renovascular disease (RVD) and RVD treated with ET-A blockers for 4 weeks. Treatment with ET-A blockers for 4 weeks significantly improved the expression of the majority of these factors, suggesting recovery of the renal angiogenic signaling, decreased microvascular and cell damage, inflammation, and fibrogenesis.*$p<0.05$ vs. Normal, $^{\dagger}p<0.05$ vs. RVD.

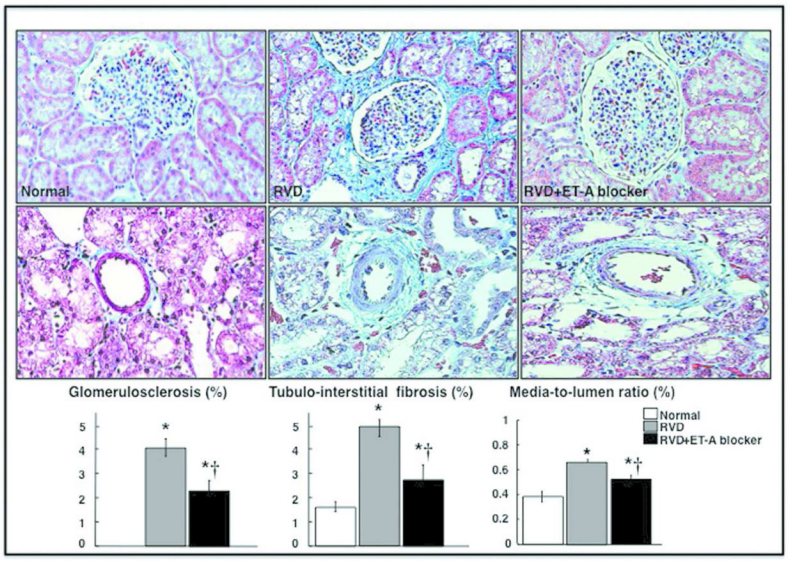


Figure 5.

Representative trichrome pictures (from stenotic kidneys) of the glomeruli (top row, x20, showed as examples to illustrate renal damage), microvessels (x40 middle row, showed as examples to illustrate renal microvascular media-to-lumen ratio), quantification of tubule-interstitial fibrosis (bottom row-left), glomerulosclerosis (bottom row-middle), and media-to-lumen ratio (bottom row-right) in normal, renovascular disease (RVD), and RVD+ET-A kidneys. Chronic ET-A blocker therapy for 4 weeks for reduced renal fibrosis and microvascular remodeling in the stenotic kidney. *$p<0.05$ vs. Normal, $^{\dagger}p<0.05$ vs. RVD.

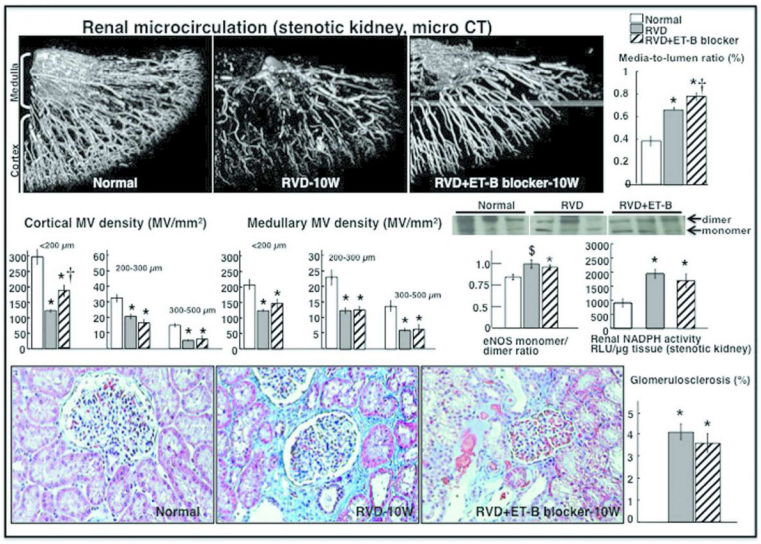


Figure 6.

Top: Representative 3D micro-CT reconstruction and quantification of microvascular media-to-lumen ratio (bottom). Middle: Quantification of the cortical and medullary microvascular density (diameters under 500 μM), renal expression of uncoupled endothelial nitric oxide synthase (eNOS, n=5–7 per group, 3 representative bands per animal shown), and NADPH-stimulated renal superoxide production in the stenotic kidney. **Bottom**: Representative trichrome pictures (from stenotic kidneys) of the glomeruli (top row, x20, showed as examples to illustrate renal damage) and quantification of glomerulosclerosis in kidneys from normal, renovascular disease (RVD), and RVD after 4 weeks of ET-B blockers. Blockade of the ET-B receptors improved (but not normalized) cortical microvascular density, but did not improve medullary microvascular density, did not reduce uncoupling of eNOS, superoxide anion, or glomerulosclerosis, and worsened microvascular remodeling in the stenotic kidney. *p<0.05 vs. Normal, †p<0.05 vs. RVD, $ p=0.07 vs. Normal.

Table 1

Mean arterial pressure, degree of stenosis, plasma renin activity, and basal single-kidney hemodynamics and function (mean ± SEM), in normal, RVD, and RVD pigs before treatment with endothelin-A (ET-A) receptor blocker (RVD+ET-A). Parameters were obtained after 6 weeks of observation.

Parameter	Normal n=7	RVD n=7	RVD+ET-A n=7
Body weight (kg)	49.6±2.6	47.7±1.4	50.0±2.1
Mean arterial pressure (mmHg)	94.6±2.8	139.1±9.1*	137.6±6.1*
Degree of stenosis (%)	0.0±0.0	72.7±9.7*	75.6±8.2*
Plasma renin activity (ng/mL/h)	0.18±0.05	0.20±0.07	0.22±0.04
Serum creatinine (μmol/L)	61.6±7.2	117.8±6.3*	125.2±7.7*
Renal vascular resistance (mmHg/mL/min)	0.18±0.05	0.49±0.13*	0.51±0.11*
Renal volume (cc)			
Cortex	127.1±7.9	66.2±6.4*	64.2±4.2*
Medulla	36.8±2.3	21.8±3.3*	22.4±2.7*
Renal blood flow (mL/min)	526.1±59.2	279.7±69.6*	265.9±55.6*
Perfusion (mL/min/cc)			
Cortex	3.3±0.4	3.1±0.7	3.4±0.4
Medulla	2.6±0.3	2.7±0.5	1.8±0.7
Glomerular filtration rate (mL/min)	51.8±2.9	39.7±7.8*	41.3±5.1*

*p<0.05 vs. Normal;

†p<0.05 vs. RVD

Table 2

Mean arterial pressure, degree of stenosis, plasma renin activity, basal single-kidney hemodynamics and function, and renal activity of superoxide dismutase (mean ± SEM), in normal, RVD, and RVD pigs treated with endothelin-A (ET-A) receptor blocker (RVD+ET-A) for 4 weeks. Parameters were obtained after 10 weeks of observation.

Parameter	Normal n=7	RVD n=7	RVD+ET-A n=7
Body weight (kg)	58.9±4.6	59.3±1.8	60.4±1.0
Mean arterial pressure (mmHg)	91.6±6.3	145.3±3.1*	127.4±2.1*†
Degree of stenosis (%)	0.0±0.0	72.7±9.7*	75.6±8.2*
Plasma renin activity (ng/mL/h)	0.19±0.03	0.22±0.03	0.21±0.05
Renal vascular resistance (mmHg/mL/min)	0.16±0.1	0.49±0.05*	0.31±0.04†^
Serum creatinine (μmol/L)	64.8±6.9	146.7±16.3*^	121.7±2.7*
Renal volume (cc)			
Cortex	130.3±6.3	64.9±9.8*	90.1±8.5*†
Medulla	39.7±1.9	19.1±2.5*	26.8±2.9*†
Renal blood flow (mL/min)	586.3±43.1	298.8±43.3*	404.7±55.6*†^
Perfusion (mL/min/cc)			
Cortex	3.8±0.4	3.7±0.7	3.7±0.7
Medulla	2.5±0.4	2.0±0.6	2.3±0.5
Glomerular filtration rate (mL/min)	55.8±3.1	40.9±7.5*	53.3±3.1^
Superoxide dismutase activity (%)	92.8±1.0	77.8±2.3*	88.7.3±4.9†

*p<0.05 vs. Normal;

†p<0.05 vs. RVD;

^p<0.05 vs. 6 weeks

Table 3

Mean arterial pressure, degree of stenosis, and basal single-kidney hemodynamics and function (mean ± SEM) in RVD pigs before (at 6 weeks) and after 4 weeks of treatment with endothelin-B (ET-B) receptor blocker (RVD+ET-B).

Parameter	RVD (6 wks) n=5	RVD+ET-B (10 wks) n=5
Mean arterial pressure (mmHg)	136.9±5.3*	148.2±6.1*
Degree of stenosis (%)	76.3±8.1*	76.3±8.1*
Renal vascular resistance (mmHg/mL/min)	0.74±0.13*	0.55±0.12††
Serum creatinine (μmol/L)	96.7±5.3*	111.1±9.3*
Renal volume (cc)		
Cortex	59.7±7.5*	64.8±7.4*
Medulla	18.4±2.4	18.2±1.1*††
Renal blood flow (mL/min)	182.8±42.3*	267.9±50.3*††
Perfusion (mL/min/cc)		
Cortex	2.7±0.6	3.6±0.4
Medulla	1.4±0.9	1.5±0.3*
Glomerular filtration rate (mL/min)	33.1±4.4*	43.5±6.2*
Superoxide dismutase activity (%)		69.4.5±6.4*

* p<0.05 vs. Normal;

† p<0.05 vs. RVD;

†† p<0.05 vs. RVD+ET-A; RVD;

^ p<0.05 vs. 6 weeks