



Published in final edited form as:

*Kidney Int.* 2013 October ; 84(4): 767–775. doi:10.1038/ki.2013.144.

## Angiotensin receptor blockade has protective effects on the post-stenotic porcine kidney

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### Abstract

Angiotensin converting enzyme inhibitors (ACEI)/ angiotensin-II receptor blockers (ARBs) may induce an acute decrease of glomerular filtration rate (GFR) in the stenotic kidney in renal artery stenosis, but most patients tolerate these drugs well. We hypothesized that ACEI/ARBs stabilize stenotic kidney function during prolonged treatment by conferring protective effects. We tested this in control domestic pigs and pigs with renal artery stenosis untreated or treated with valsartan, or triple therapy (7 pigs in each group) for 4 weeks starting 6 weeks after stenosis induction. Renal function, oxygenation, tubular function, and microcirculation were assessed by multi-detector computed tomography (CT), blood-oxygen-level-dependent magnetic-resonance imaging, and micro-CT. Valsartan and triple therapy decreased blood pressure similarly, however, valsartan did not change the GFR of the stenotic kidney compared to renal artery stenosis and was similar to triple therapy. Both valsartan and triple therapy stimulated microvascular density, and improved tubular function. Valsartan also caused a greater increase of angiogenic factors and a decrease in oxidative stress, which were related to higher cortical perfusion and tubular response than triple therapy. Thus, valsartan did not decrease stenotic kidney GFR, but improved cortical perfusion and microcirculation. These beneficial effects may partly offset the hemodynamic GFR reduction in renal artery stenosis and preserve kidney function.

### Keywords

Renal artery stenosis; angiotensin II type I receptor blockade; microvasculature

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**DISCLOSURE** All the authors declared no competing interests.

## INTRODUCTION

The prevalence of renal artery stenosis (RAS) is fast-growing world-wide, especially in the elderly population. In addition to hypertension and progressive renal function decline, the incidence of cardiovascular morbidity and mortality is also on the rise.<sup>1</sup> These pathophysiological changes are accompanied by increased levels of Angiotensin II (AngII) which is formed by activated renin-angiotensin-aldosterone system (RAAS) in RAS. The application of angiotensin converting enzyme inhibitors (ACEI) or Ang II receptor blocker (ARB), which block the effects of AngII by binding to its type I receptor (AT1), have conferred invaluable benefits not only in relieving renovascular hypertension, but also lowering the cardiovascular complications.<sup>2, 3</sup>

In the kidney, although the inhibition of RAAS has been useful in alleviating proteinuria and slowing renal function decline, it might acutely reduce the stenotic kidney capillary hydrostatic pressure and consequently glomerular filtration rate (GFR).<sup>4, 5</sup> However, abundant evidence has shown that in most patients these drugs are well tolerated.<sup>6</sup> Conceivably, chronic blockade of RAAS might also blunt deleterious processes in the kidney, such as microvascular remodeling that characterizes ischemic kidney damage,<sup>7, 8</sup> and may modulate oxidative stress and inflammation as observed in other forms of CKD.<sup>9, 10</sup>

However, the potential effects of long-term ARB on the microvasculature within the stenotic kidney remain unclear. This study tested the hypothesis that chronic treatment with the ARB Valsartan would preserve stenotic kidney function in a unilateral RAS swine model, similar to a conventional antihypertensive regimen, by decreasing tissue injury that may offset the hemodynamic reduction of GFR.

## RESULTS

### 1. Animal Characteristics

At 10 wks, there were no differences in body weight and degree of stenosis among the groups (Table 1), including Normal, unilateral RAS, RAS+Valsartan or RAS+triple therapy (TT, reserpine+hydralazine+hydrochlorothiazide). Blood pressure changes of the study groups during the observation are shown in Figure 1-1. Mean arterial pressure (MAP) increased at 1 wk after RAS induction and remained similarly elevated at 6 wks ( $P=0.29$  among 3 groups), suggesting hemodynamically significant RAS. MAP continued increasing in untreated RAS at 10wks ( $P<0.05$  vs. 6wks), but was lowered by both Valsartan and TT groups to the same level, although neither reduced MAP to normal ( $P=0.02$  and  $0.04$  vs. Normal, respectively). Urine protein was increased in all 3 RAS groups, but was alleviated only by Valsartan ( $P=0.040$  vs. RAS,  $P=0.053$  vs. Normal, Table 1). Valsartan also decreased urinary levels of the kidney injury marker neutrophil gelatinase-associated lipocalin (NGAL) that was elevated in RAS. Creatinine level was increased in all RAS groups, and unaffected by either treatment. Serum potassium in RAS+Valsartan group did not differ from Normal and RAS, while TT induced a slight decrease. Serum sodium was comparable among the groups.

## 2. Renal function and oxygenation

Stenotic-kidney GFR was decreased in all RAS groups, as were renal blood flow (RBF) and cortical renal perfusion (Figure 1–2, A–C). Neither Valsartan nor TT increased RBF or GFR, yet cortical perfusion was slightly but significantly increased in RAS+Valsartan compared to RAS.

Basal medullary R2\*, an index of the concentration of deoxyhemoglobin in the kidney,<sup>11, 12</sup> in both the RAS+Valsartan and RAS+TT groups was higher than Normal (Figure 1–3B), suggesting lower medullary oxygenation. The tubular response to furosemide, which decreases R2\* through increasing medulla oxygenation via inhibition of solute transport and thereby of oxygen consumption,<sup>12</sup> was abolished in RAS, but was improved by TT and to a slightly greater extent, by Valsartan (Figure 1–3C), indicating recovery of viability of medullar tubules. Cortical R2\* value was slightly lower in RAS+Valsartan than in RAS, consistent with lower levels of cortical deoxyhemoglobin (Figure 1–3A).

## 3. Intra-renal microvasculature

RAS decreased the density of both small and medium-size microvessel through the inner and outer cortex (Figure 2–1 A–D, E–F). Valsartan normalized outer cortical small vessels density and improved that of medium-size vessels (P=0.01 vs. RAS). In contrast, TT improved only small vessels density. Microvascular density in the inner cortex remained unchanged by either drug.

Consistent with the microvascular density changes, Valsartan increased expression of basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), and normalized the expression of its receptor-2 (FLK-1) that was suppressed in RAS (Figure 2-2, A). TT only increased the receptor FLK-1 expression (P=0.01 vs. Normal). Expression of endothelial nitric oxide synthase (eNOS) remained unchanged among the groups. Valsartan also enhanced the expression of the neovessel maturation/stability modulators<sup>13</sup> angiopoietin-1 and its receptor Tie-2, which was down regulated in RAS. TT also improved expression of Tie-2, but to a lesser extent than Valsartan. Therefore, Valsartan promoted both angiogenic factors and their receptors, but TT mainly activated their receptors. Notably, TT but not Valsartan increased thrombospondin (TSP)-1 (P=0.01 vs. Normal), which inhibits neovascularization. Hence, both Valsartan and TT improved microvascular density, but Valsartan tended to exert greater angiogenic impact. Hypoxia-inducible factor (HIF)-1 alpha, closely linked to neovascularization in tissue ischemia, was down-regulated in RAS, as previously reported in our RAS model,<sup>14</sup> but normalized by both medication regimens (Figure 2-2, B)

## 4. Oxidative stress, inflammation and fibrosis

Dihydroethidium (DHE) staining indicated increased oxidative stress in RAS that was attenuated by Valsartan (P=0.01 vs. RAS) but not TT (P=0.07 vs. Normal). This was consistent with NAD(P)H oxidase P47 expression, which was increased in RAS, but decreased only by Valsartan (Figure 3–1). Tumor necrosis factor (TNF)-alpha expression in RAS kidneys was enhanced in renal tubules and interstitium, but was attenuated to similar extent by Valsartan and TT (Figure 3–2). Trichrome staining showed that fibrosis was

blunted to the same extent by both Valsartan and TT compared to RAS (Figure 3-3). In line with trichrome staining, both Valsartan and TT normalized elevated matrix metalloproteinases (MMP)-2 expression in RAS and down-regulated tissue-inhibitor of metalloproteinases (TIMP)-1. TT also induced a greater decrease in transforming growth-factor (TGF)-beta than Valsartan ( $P=0.03$ ). Neither medication decreased connective tissue growth factor (CTGF) (Figure 3-4).

## 5. Ang II receptors

AT1R was upregulated in RAS, and Valsartan further enhanced its expression, possibly as a feedback to its blockade. AT2R was similarly elevated in all RAS groups (Figure 4).

## DISCUSSION

Our study shows that for the same level of blood pressure control, a 4-week chronic treatment with the ARB Valsartan achieved similar renal function and did not decrease the stenotic kidney GFR compared to a conventional antihypertensive regimen. In addition, Valsartan elicited a slightly better preservation of intra-renal perfusion, microvascular density, and medullary tubular function, although overall kidney function was similar to that achieved with TT. Nonetheless, our data suggest that the safety and efficacy of Valsartan in the management of renovascular hypertension in RAS might be mediated by a novel protective effect on the intrarenal microvasculature.

An important mechanism contributing to intrarenal damage distal to RAS, microvascular remodeling and regression characterize the pathophysiologic alteration caused by prolonged vasoconstriction, oxidative stress and fibrosis.<sup>7, 8, 15</sup> A physiological acute decrease in oxygen supply up-regulates the expression of HIF-1-alpha, a primary defense mechanism in the adaptive response to ischemia, and in turn of VEGF that induces new vessel formation. However, this compensatory capability can be impaired in chronic ischemia by amplified oxidative stress and inflammation in RAS (reflected in our study by increased DHE staining, P47 expression, and TNF-alpha), which destabilize HIF-1-alpha and degrade VEGF protein, thus interfering with tissue repair by compensatory angiogenesis.<sup>7, 8, 14</sup> Accordingly, the present study demonstrated that HIF-1 alpha was suppressed in RAS, along with down-regulated FLK-1 and VEGF that failed to increase. The unchanged level of VEGF could be a result of its dynamic temporal expression pattern in ischemia.<sup>16, 17</sup> Interestingly, our data show that Valsartan activates multiple angiogenic factors in the stenotic kidneys, including bFGF, VEGF/FLK-1 and HIF-1-alpha, possibly by alleviating oxidative stress and inflammation (TNF-alpha), as observed in other chronic kidney diseases.<sup>9, 10, 18</sup> Furthermore, Valsartan upregulated the key modulators of vessel stability and maturation Angiotensin-1 and Tie-2. Collectively, these effects may account for the slightly greater improvement of intra-renal microvascular density than that achieved by conventional TT, although both drug regimens improved cortical microvascular density. Such benefits are likely functionally consequential, because we have previously shown that loss of small outer cortical microvessels correlates with persistent kidney dysfunction after renal artery revascularization.<sup>19</sup> However, while TT increased the expression of the receptors FLK-1 and Tie-2 and restored HIF-1 alpha, these might have been offset by the parallel upregulation of

TSP-1. Therefore, some of the beneficial effects of Valsartan on the kidney might be mediated by a microvascular protective effect, which appears to be blood pressure-independently.

The drugs used for TT decrease blood pressure by direct vasodilation, diuresis, or central monoamine-depleting, and are considered to have few effects on inflammation, oxygenation, and the microvasculature. Importantly, we observed in this study minor but significant renal protective effects, which might be attributed to vasodilation of intra-renal vessels, because blood pressure reduction per se might further decrease renal perfusion distal to the stenosis. Indeed, hydralazine has also been linked to anti-oxidative and anti-inflammatory effects.<sup>20, 21</sup> In addition, hydrochlorothiazide might sustain GFR by increasing efferent arteriolar resistance.<sup>22</sup> A protective effect of TT has been observed in other models, yet is often ascribed to antihypertensive properties.<sup>23</sup>

We have previously shown that basal  $R2^*$  in post-stenotic human kidneys increases in severe<sup>24</sup> but not moderate ischemia,<sup>25</sup> while medullary response to furosemide is blunted in the latter, as observed in RAS pig kidneys in the current study. Presumably, decreased tubular injury and resumed transport activity, reflected by an increase of  $\Delta R2^*$  in response to furosemide, might have contributed to the increase in basal  $R2^*$  in RAS + Valsartan and RAS+TT kidneys.<sup>26</sup> Hence, our data reinforce the notion of a parallel decrease in oxygen-dependent tubular transport and subsequently oxygen consumption as a possible primary mechanism to minimize hypoxia and preserve tubular cells.<sup>26</sup> Indeed, in parallel to the increase in basal  $R2^*$  value in our study, HIF-1  $\alpha$  that was inhibited in RAS also increased by Valsartan and TT. The long-term effects of increased tubular work in the face of unchanged RBF warrant further studies, although evidently both approaches ultimately decrease renal fibrosis in our model.

ARB exert their beneficial effects via blocking the binding of AngII to the AT1 receptor, thereby allowing it to bind to the AT2 receptor.<sup>27</sup> Both the AT1 and AT2 receptors were up-regulated in RAS, as previously shown,<sup>28</sup> and we also found that AT1R expression was further increased by Valsartan, probably a feedback secondary to receptor blockade. Notably, this may also affect the microvascular remodeling observed in our study. Although microvascular rarefaction induced by oxidative stress, inflammation and fibrosis dominates kidney remodeling in RAS, direct pro-angiogenic effects of AngII include up-regulating angiogenic factors such as VEGF, bFGF, eNOS and Ang-1/Tie-2, and mediated by binding to either the AT1 or AT2 receptors.<sup>29</sup> However, the expression pattern of AT1 and AT2 receptors differs greatly by tissue, and may impact the involvement of AngII in neovascularization.<sup>30</sup> While AT1R is abundant in a number of adult tissues,<sup>31</sup> the AT2R, in general, is restricted in adults to organs such as kidney, brain and adrenal gland. Hence, the influence of ARB on angiogenesis may possibly be affected by the type of tissue.<sup>30</sup> Indeed, in our study the improvement of microvascular density after AT1-R blockade by Valsartan could be attributed to binding of AngII to the upregulated AT2-R, possibly stimulated by kidney injury.<sup>31, 32</sup> This stipulation is supported by the observation that chronic treatment with Losartan for 2 weeks increased microvessel density in the brain, where AT2 receptor is also richly expressed.<sup>33</sup>

## Limitations

Our study is limited by the use of relatively young animals and short duration of the disease, yet renal structure and function in our swine model are similar to human kidneys. The long-term effect of ARB on stenotic kidney function, and the contribution of microvascular protection to the improvement of renal function, warrant further studies. The blockade of the RAAS in our study was incomplete, as suggested by the partial blood pressure-response, indicating the need for dose adjustments in pigs. Nevertheless, the comparable decrease in blood pressure relative to TT implies that any observed effects of Valsartan compared to TT were blood pressure-independent. As TT can increase renin release and AngII, its influence on AngII and its link to microvascular change or other mechanisms of ischemic kidney injury should also be further explored.

## Conclusion

In summary, our study demonstrated that a 4-wk regimen of ARB after 10 weeks of swine unilateral RAS did not impair hemodynamics or function in the stenotic kidney, compared to untreated RAS or to conventional antihypertensive treatment eliciting a comparable blood pressure decrease. We also observed improved renal microvascular density and decreased tubulointerstitial fibrosis in the stenotic kidney of pigs treated with TT. In addition, Valsartan seems to offer slightly greater gains in cortical microvascular density and function (perfusion), and in medullary tubular function, possibly by upregulating expression of several growth factors and suppressing oxidative stress and inflammation. While the measureable differences between Valsartan and TT were not striking, they were associated with a greater decrease in urinary protein and NGAL levels, and may partly offset a hemodynamic ARB-induced reduction in GFR in the ischemic kidney. Therefore, our data imply that chronic use of ARB may not only be safe in unilateral RAS, but may provide reno-protective benefit.

## MATERIALS AND METHODS

### 1. Animal groups

This study was approved by the Institutional Animal Care and Use Committee. Twenty-eight domestic female pigs (50–60 Kg) were studied during 10 weeks of observation. Pigs were randomized to groups (n=7 each) of untreated shams (Normal), unilateral RAS (n=7), and RAS groups chronically treated with Valsartan (RAS+Valsartan, n=7) or triple therapy (RAS+TT) of Hydralazine, Reserpine and Hydrochlorothiazide (RAS+TT, n=7). Pigs were fed isocaloric diets of standard chow containing 0.15–0.65% sodium chloride (NaCl), and had free access to water.

### 2. RAS induction

RAS was induced at baseline by placing a local irritant coil in the right main renal artery, leading to gradual development of unilateral RAS, as previously described.<sup>34</sup> A telemetry transducer was then implanted in the left femoral artery<sup>35, 36</sup> to continuously monitor blood pressure for the following 10 wks. Six wks later, the degrees of stenosis were determined by renal angiography.

### 3. Drug treatment

After determination of the degree of stenosis at 6 weeks of RAS, antihypertensive drugs were fed with food in the corresponding groups for 4 additional weeks. Valsartan (Molecular Weight 435.5, generously provided by Novartis Pharmaceuticals Corporation, NJ), was delivered at 320mg daily, as previously safely used in swine.<sup>37, 38</sup> The dose of TT components was adjusted weekly based on the blood pressure change, in order to achieve comparable blood pressure with Valsartan group. TT was started with Reserpine 0.1mg/day, hydralazine 25mg/day, and hydrochlorothiazide 12.5mg/day, and reached an average dose at 10 wks of Reserpine 0.2mg/day, Hydralazine 46mg/day, and Hydrochlorothiazide 21mg/day.

### 4. In Vivo Studies

**1) Multi-Detector Computed Tomography (MDCT)**—At 10 wks, 4 wks after initiation of drug treatment, MDCT scanning was performed to assess in each stenotic (or normal) kidney renal blood flow (RBF) and GFR, as described previously.<sup>34, 39</sup> Briefly, 160 consecutive scans were performed following a central venous injection of iopamidol (0.5 ml·kg<sup>-1</sup>·2s<sup>-1</sup>). Then, MDCT images were reconstructed and displayed with the Analyze® software package (Biomedical Imaging Resource, Mayo Clinic, Rochester, MN).

For data analysis, regions of interest (ROI) were selected from tomographic images from the aorta, renal cortex, and medulla to generate time-attenuation curves in each region and obtain measures of renal function.<sup>39</sup>

**2) Blood oxygen level dependent (BOLD)-magnetic resonance imaging (MRI)**—Two days before MDCT, 3T BOLD MRI (Signa Echo Speed; GE Medical Systems, Milwaukee, WI) scanning was performed to assess intra-renal oxygenation, evaluated as R2\*<sup>11, 12</sup>.

To further examine the changes in medullary oxygen consumption as a result of solute transport, BOLD measurements were repeated 15 min after a furosemide (20 mg) injection into an ear vein cannula. Furosemide increases medullary oxygenation through inhibition of solute transport and thereby of oxygen consumption.

For data analysis, ROIs were manually traced in the cortex and medulla<sup>11, 12</sup>. For each echo time, the software automatically computed the average of MR signals within each ROI. The BOLD signal (R2\*) was measured, and its change after furosemide defined as “delta-R2\*”.

Animals were euthanized three days later after MDCT scanning, using a lethal intravenous dose of sodium pentobarbital (Sleepaway®, 100mg/kg, Fort Dodge Laboratories, Fort Dodge, IA). The stenotic kidneys were removed using a retroperitoneal incision and immediately dissected and preserved in ice-cold normal saline for micro-CT, frozen in liquid nitrogen (and maintained at -80°C), or preserved in formalin for tissue studies.

### 5. In Vitro Studies

**1) Blood and urine sample**—Blood samples were taken from the inferior vena cava at the time of MDCT studies, centrifuged at 5000 rpm for 10 min, and kept at -80°C until

determination of creatinine (spectrophotometry), sodium, and potassium. Urinary protein and Neutrophil gelatinase-associated lipocalin (NGAL), a marker of kidney injury<sup>40</sup> were measured to probe kidney damage.

**2) Micro CT**—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 the renal artery. Samples were prepared and scanned at 0.5° angular increments at 18- $\mu$ m resolution, and images analyzed as previously described.<sup>7, 35</sup> The spatial density of microvessels (defined as diameters <500  $\mu$ m) in the inner and outer renal cortex were calculated and classified according to diameter as small (<40  $\mu$ m), medium (40–200  $\mu$ m) or large (200–500  $\mu$ m) microvessels.<sup>7</sup>

**3) Histology**—In-situ renal production of superoxide anion was evaluated by DHE staining. Immunoreactivity of TNF-alpha (1:200, NOVUS) was assessed for intra-renal inflammation and renal fibrosis by trichrome staining. Imaging analysis was performed using a computer-aided image-analysis program (AxioVision® v4.7.2.0, Carl Zeiss MicroImaging, Thornwood, NY).

**4) Protein expression and western blotting**—Standard blotting protocols were followed, using specific polyclonal antibodies against VEGF (1:200 Santa-Cruz), FLK-1 (1:200 Santa-Cruz), bFGF (1:1000 abcam), eNOS (1:1000 abcam), angiopoietin-1 (1:200 Santa-Cruz) and its receptor Tie-2 (1:1000 abcam), the TSP-1 (1:500 abcam), and HIF-1 alpha (1:1000 abcam) for angiogenesis mechanisms, NAD(P)H oxidase P47 (1:200 Santa-Cruz) for oxidative stress, MMP-2, TIMP-1, TGF-beta (all 1:200 Santa-Cruz) and CTGF (1:500 abcam) for evaluation of fibrosis, and AT1 (1:200 Santa-Cruz) and AT2 (1:800 abcam) receptors.

Horseradish peroxidase secondary antibodies (GE Healthcare UK Limited) were used and chemiluminescence determined using the SuperSignal West Pico Chemiluminescent Substrate or SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, IL) according to vendor's instructions. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:5000 Covance, Emeryville, CA) served as loading control. Intensities of the protein bands (one per animal) were quantified using densitometry by Alpha-Imager 3400 software (Alpha Innotech Corp., San Leandro, CA).

## 6. Statistical Analysis

Continuous data are expressed as mean  $\pm$  SEM. Comparisons within groups were performed using the paired Student's *t*-test and among groups ANOVA and unpaired *t*-test.  $P < 0.05$  was considered statistically significant.

## Acknowledgements

This work was partly supported by the National Institutes of Health (HL085307, DK73608 and HL77131).



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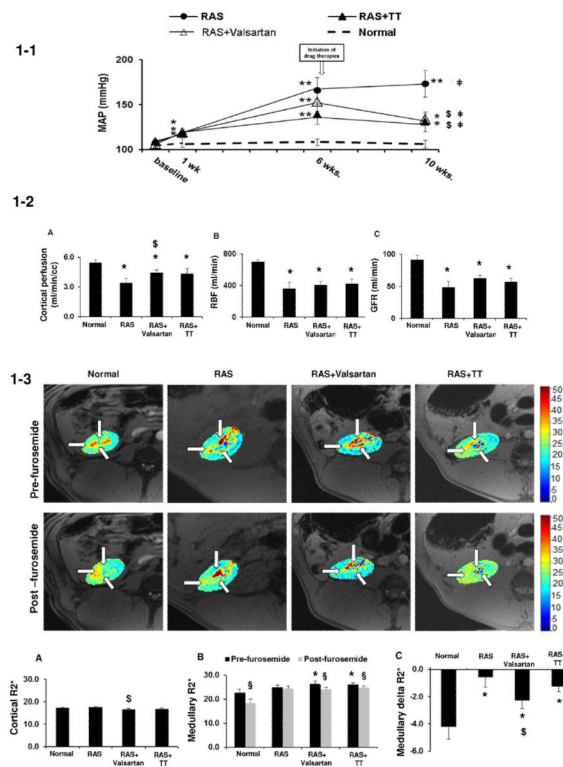
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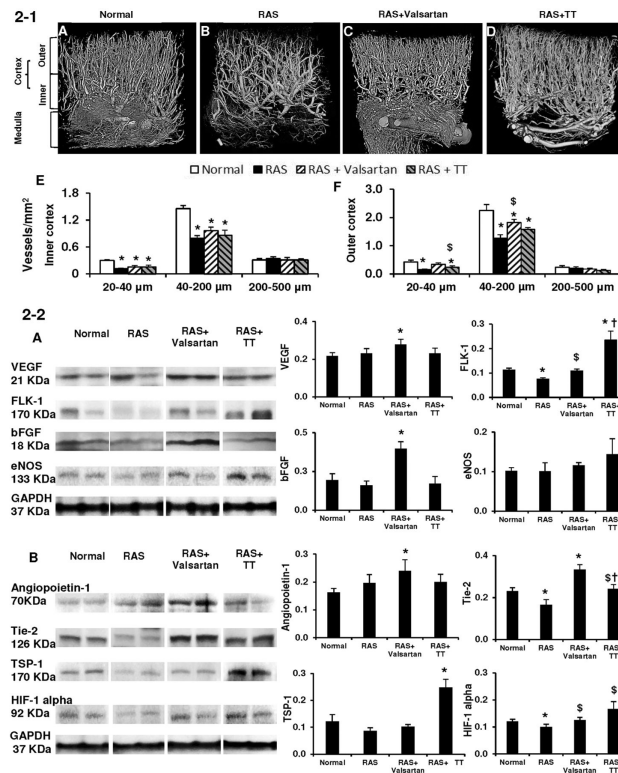
**Figure 1.**

**1-1** Mean arterial pressure (MAP) assessed by telemetry over 10 weeks of observation in Normal, renal artery stenosis (RAS), RAS+Valsartan, and RAS+triple therapy (TT). MAP increased 1 week after induction of RAS and remained elevated in untreated RAS for the duration of the study. Valsartan and TT similarly lowered, but not normalized, MAP.

**1-2: A–C** Perfusion, renal blood flow (RBF) and glomerular filtration rate (GFR) assessed by MDCT at 10 weeks. All RAS groups had lower perfusion, RBF, and GFR compared to Normal, but Valsartan improved cortical perfusion compared to untreated RAS.

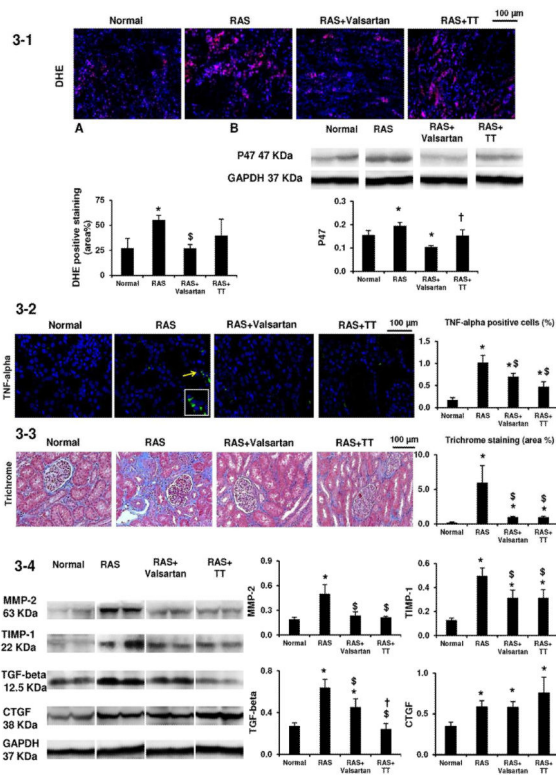
**1-3** Representative images of stenotic kidney blood oxygen level dependent (BOLD) MRI before (top) and after (bottom) furosemide injection, and R2\* in the cortex (A) and medulla (B) before and after furosemide. C: Delta-R2\* in medulla. Both Valsartan and TT restored medullary tubular response to furosemide, but Valsartan elicited a greater improvement than TT. Arrows (white) indicate the approximate size of the medullary regions.

\*P<0.05 vs. Normal; \*\*P<0.01 vs. Normal; \$P<0.05 vs. RAS; ‡ P<0.05 vs. 6 wks; § P<0.05 vs. Pre-furosemide.

**Figure 2.**

**2-1 A-D** Representative 3-dimensional images of the renal cortical microvasculature in Normal, renal artery stenosis (RAS), RAS+Valsartan and Triple therapy (TT) pig kidneys. **E-F**: quantification in the outer and inner cortex. RAS induced loss of small (20–40μm) and medium (40–200μm) microvessels in both the outer and inner cortex. TT improved small vessels in the outer cortex, while Valsartan normalized small vessels and improved medium-size vessels as well.

**2-2 A-B** Renal expression of angiogenic and angiostatic markers. Both Valsartan and TT stimulated angiogenesis, but Valsartan stimulated more angiogenic factors, while TT also upregulated the angiogenic inhibitor. Protein bands were quantified relative to GAPDH. Two representative bands from each group are shown. \* P<0.05 vs. Normal; \$ P<0.05 vs. RAS; †P<0.05 vs. RAS+Valsartan.

**Figure 3.**

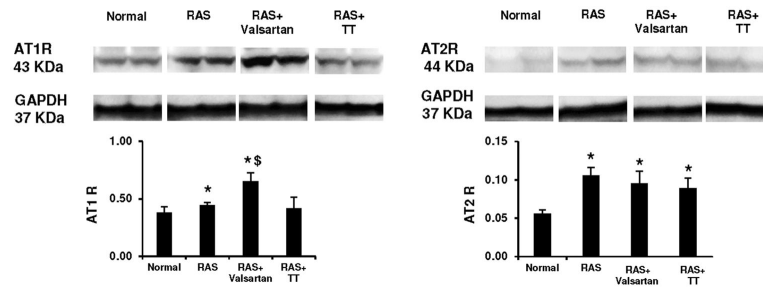
**3-1** Representative dihydroethidium (DHE) images [40 $\times$ , positive DHE staining (pink), nuclei (blue)], its quantification (A) and renal protein expression of P47 (B). Renal oxidative stress was increased in renal artery stenosis (RAS) and restored only by Valsartan.

**3-2.** Representative tumor necrosis factor (TNF)-alpha staining images [40 $\times$ ] and quantification. TNF-alpha positive cells (green, blue nuclei) were increased in the renal tubules and interstitium of RAS pigs. Both Valsartan and triple therapy (TT) attenuated TNF-alpha expression.

**3-3.** Representative trichrome staining (20 $\times$ ) and quantification. Both Valsartan and TT alleviated fibrosis compared to RAS.

**3-4.** Renal expression of fibrogenic markers. Valsartan and TT showed a similar anti-fibrotic effect.

Protein bands were quantified relative to GAPDH. Two representative bands per group are shown. \*  $P < 0.05$  vs. Normal; \$  $P < 0.05$  vs. RAS; †  $P < 0.05$  vs. RAS+Valsartan.



**Figure 4.** Renal expression of angiotensin receptors AT1R and AT2R. Valsartan alone increased AT1R, while AT2R was increased in all RAS groups. Protein bands were quantified relative to GAPDH. Two representative bands from each group are shown. \*P<0.05 vs. Normal; \$ P<0.05 vs. RAS.

**Table 1**

Animal systemic characteristics in normal, unilateral renal artery stenosis (RAS), and Valsartan or triple therapy (TT)-treated RAS for 4 weeks.

Characteristics	Normal (n=7)	RAS (n=7)	RAS+Valsartan (n=7)	RAS+TT (n=7)
Body Weight (kg)	49.4±0.8	50.8±2.1	48.3±1.9	47.8±0.9
Degree of stenosis (%)	-	78 ± 7	76 ± 4	78 ± 5
MAP (mmHg)	106.0±4.1	173.4±5.0*	134.0±6.3*,\$	130.9±11.2*,\$
Urine protein (µg/ml)	13.2±1.3	40.2±5.6*	23.8±4.4\$	29.7±5.0\$*
Urine NGAL (ng/ml)	6.08±0.26	8.32±0.71*	6.62±0.21\$	7.21±0.47
Serum Creatinine (mg/dl)	1.23±0.04	1.60±0.08*	1.52±0.05*	1.73±0.03*
Serum K (mmol/l)	3.78±0.11	3.75±0.14	3.57±0.19	3.38±0.10*
Serum Na (mg/dl)	138.8±0.9	136.0±2.0	137.7±5.1	139.8±0.8

Data were expressed as mean±SEM.

RAS: renal artery stenosis. TT: triple therapy. MAP: mean arterial pressure. NGAL: Neutrophil gelatinase-associated lipocalin.

\* P<0.05 vs. Normal;

\$ P<0.05 vs. RAS.