Regression of Renal Disease by Angiotensin II Antagonism Is Caused by Regeneration of Kidney Vasculature

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ABSTRACT
Chronic renal insufficiency inexorably progresses in patients, such as it does after partial renal ablation in rats. However, the progression of renal diseases can be delayed by angiotensin II blockers that stabilize renal function or increase GFR, even in advanced phases of the disease. Regression of glomerulosclerosis can be induced by angiotensin II antagonism, but the effect of these treatments on the entire vascular tree is unclear. Here, using microcomputed tomography and scanning electron microscopy, we compared the size and extension of kidney blood vessels in untreated Wistar rats with those in untreated and angiotensin II antagonist–treated Munich Wistar Frömter (MWF) rats that spontaneously develop kidney disease with age. The kidney vasculature underwent progressive rarefaction in untreated MWF rats, substantially affecting intermediate and small vessels. Microarray analysis showed increased Tgf-β and endothelin-1 gene expression with age. Notably, 10-week inhibition of the renin-angiotensin system regenerated kidney vasculature and normalized Tgf-β and endothelin-1 gene expression in aged MWF rats. These changes were associated with reduced apoptosis, increased endothelial cell proliferation, and restoration of Nrf2 expression, suggesting mechanisms by which angiotensin II antagonism mediates regeneration of capillary segments. These results have important implications in the clinical setting of chronic renal insufficiency.


The progression of renal diseases leads invariably to end stage organ failure in patients as well as experimental models. This translates into one half million patients reaching ESRD each year globally and >700,000 deaths. The scale of the problem has been underestimated, whereas the costs for RRT are becoming problematic, even for wealthy nations. In the last 10 years, we and others have provided evidence that this progression can be significantly delayed and even reversed in rats and humans by the use of drugs that interfere with the renin-angiotensin system (RAS). However, despite important clinical implications, the mechanisms by which RAS inhibition induces reversal of renal lesions have not been established so far.

In this study, using imaging of rat kidney by microcomputed tomography (microCT) and scanning electron microscopy (SEM), we analyzed extension and organization of micro- and macrovasculature in Munich Wistar Frömter (MWF) rats, a model of progressive glomerular injury, and normal control Wistar rats. We also investigated the effect of RAS inhibition on kidney vasculature in MWF rats, treatments known to induce regression of renal structural and functional changes that develop spontaneously with age in these animals. Other than morphologic and morphometric evaluations of kidney vasculature, we finally elucidated the potential molecular mechanisms by gene and protein expression that could account for loss of vascular segments and changes in glomerular capillary (GC) organization in MWF rats and successful regeneration of kidney vasculature obtained by RAS inhibition.

We investigated kidney vasculature in normal Wistar and MWF rats at different ages and with different treatments as reported in the diagram in Figure 1A. In MWF rats, the spatial density of arteries

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and veins was importantly reduced as kidney disease progressed at 50 weeks of age, when histologic damage at glomerular and tubular levels was already marked (Supplemental Figure 1, Supplemental Table 1) and important proteinuria developed, compared with Wistar rats (444±89 versus 6.6±1.5 mg/24 h in MWF50 versus Wistar50, respectively). The same pattern was observed for small arterial and venous vessels as well as the microcirculation (Figure 1B). Thus, the volume density of intermediate-sized vessels (80–180 μm diameter) was significantly reduced in aged MWF rats compared with controls (Figure 1, B and C). Both angiotensin–converting enzyme inhibitor (ACEi) and angiotensin II receptor antagonist (AIIRA) treatments markedly improved renal histology (with regression of glomerular and tubular damage) (Supplemental Figure 1, Supplemental Table 1), significantly reduced proteinuria (499±216 MWF60, 69±36 MWF+LIS60, and 101±42 mg/24 h MWF+LOS60), and robustly regenerated kidney vasculature (Figure 1D). Of note, both treatments importantly increased vasculature extension (Figure 1, C and D), which was higher than in control animals at same age. The regeneration of new vascular segments is also shown by the statistically significant increase in estimated length of small caliber blood vessels (Figure 1E). We also verified (as reported in Supplemental Material) that angiotensin–converting enzyme inhibition did not affect the volume density and estimated capillary length in two additional groups of Wistar rats (data not shown). Vascular rarefaction in MWF rats and regeneration after RAS inhibition were also confirmed by evaluating capillary volume density with rat endothelial cell antigen (Recal) staining in both interstitial and glomerular areas (Figure 1F).

Vascular rarefaction is expected to translate in GC injury, which resulted in proteinuria, glomerulosclerosis, and

![Figure 1. RAS blockade regenerates kidney vasculature. (A) Diagram of the experimental design. (B) Representative three–dimensional views of kidney vasculature by ex vivo microCT in 50- and 60-week-old MWF rats compared with 50-week-old Wistar rats and representation of intermediate– and small–sized capillary beds. (C) Quantification of vascular and kidney volume (top panel), vessels with diameter ranging from 80 to 180 μm (middle panel), and vessels with diameter <80 μm (bottom panel). (D) Representation of kidney vasculature in MWF rats on treatment with lisinopril and losartan. (E) Equivalent vessel length of blood vessels with diameters ranging from 80 to 180 μm (upper panel) and <80 μm (lower panel). (F) Quantification of capillary volume density (Vv) as a percentage of Reca1 positivity in interstitial (upper panel) and glomerular (lower panel) areas. Values are means±SDs; n=5 for Wistar and MWF50 rats, n=10 for untreated MWF60 rats and MWF rats treated from 50 to 60 weeks of age with lisinopril, and n=7 for MWF rats treated from 50 to 60 weeks of age with losartan. *P<0.05 versus MWF60; **P<0.01 versus MWF60; ***P<0.05 versus MWF50; ****P<0.01 versus MWF50. Lis, lisinopril; Los, losartan; Reca1, rat endothelial cell antigen 1.](image-url)
loss of kidney function.\(^5\) We also reported that RAS inhibition induced regression of glomerular lesions and enhanced the tuft area devoid of sclerotic lesions.\(^4\)

Here, we have now characterized the effects of ACEi and AIIRA on aged MWF rats at the level of GC ultrastructure by virtue of quantitative SEM. As shown in Figure 2A (and more extensively in Supplemental Figure 2), major changes characterized the glomerular population in MWF rats. Thus, mean GC volume \((V_G)\) increased with age compared with that in Wistar rats, with remarkable broadening of \(V_G\) distribution (Figure 2B), likely for a compensatory mechanism. Similarly, the shape of the GC tuft in MWF rats was more irregular compared with that in Wistar rats, which was shown by the distribution of the circularity parameter (Figure 2C). Morphometric estimation of GC length (Figure 2D) showed a more heterogeneous distribution in the MWF50 group, with some glomeruli characterized by very long (up to 32 mm) and short (<3 mm) capillaries. Of interest, the mean GC diameter was higher in MWF rats compared with Wistar rats (10.9±0.3 versus 7.4±0.4 μm). RAS inhibition in MWF rats by lisinopril did not reduce but actually, increased the glomerular enlargement and heterogeneity observed with age in these rats. Actually, \(V_G\) was higher in lisinopril–treated MWF rats than in untreated animals, with a higher number of glomeruli with large volume (up to \(8 \text{ μm}^3\times10^{-6}\)). The same was observed for GC length (median [range]; 22.7 [4–51] in MWF +LIS60 versus 17.6 [3–61] mm in MWF60), with a larger fraction of glomeruli characterized by longer capillary length. On the contrary, capillary diameter remained constant (9.8±0.7 versus 9.4±0.6 μm). This detailed analysis of GC structure suggests that the kidney disease in this model modifies (to a major extent) the structure of functioning glomerular capillaries and ACEi treatment, likely affecting the remaining functional nephrons and further increasing the heterogeneity of the glomerular tufts with formation of new capillary segments in already enlarged capillary tufts. These changes likely provide higher volumes of functional GC segments and consequently, increased filtering surface area. Our results of increased mean diameter as well as segment length indicate that hydraulic resistance of GC networks may not be changed by treatments. Actually, enlargement of capillary diameter is expected to reduce hydraulic resistance, whereas elongation of capillary segments is expected to increase it. These two effects may cancel each other.

To clarify the molecular mediators underlying the regeneration of the kidney vasculature elicited by RAS blockade, we first analyzed by PCR array the renal expression profile of 84 genes involved in the modulation of the biologic processes of angiogenesis (Figure 3A). Unexpectedly, gene expression of vascular growth–promoting factors, such as Vegf and related receptors or angioptinetin-1 and -2, did not change between MWF60 and Wistar rats, whereas genes related to fibrosis, inflammation, and extracellular matrix remodeling were differentially expressed between the two strains. This framework is consistent with hallmarks of renal scarring that characterize advanced nephropathy in MWF rats when vasculature rarefaction was strongly evident. Upregulated profibrotic genes included the three Tgf-β isoforms, with Tgf-β2 being the most expressed, and endothelin-1 (Et-1). Validation of the array data by quantitative RT-PCR (Figure 3B) confirmed renal overexpression of Tgf-β2 and Et-1 genes in MWF rats. Of interest is the observation that abnormal expression of such genes was almost normalized by both ACEi and AIIRA. These findings are in line with previous evidence showing that renoprotection induced by ACEi in MWF rats was associated with normalization of TGF-β protein in glomeruli and the cortical interstitium and paralleled the reduction in urinary excretion of ET-1, which likely reflects the renal synthesis of the peptide.\(^4,6,7\) ET-1, synthesized

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**Figure 2.** ACE inhibition enhances GC volume and length. (A) Representative SEM images of GC lumen in Wistar and MWF rats. (B) \(V_G\) distribution histograms in Wistar and MWF rats. (C) Distribution histogram of glomerular circularity. (D) Distribution histogram of estimated individual capillary length. Values are median (range; \(n=225\) glomeruli per group). **\(P<0.01\) versus MWF60; ***\(P<0.01\) versus MWF50. LIS, lisinopril.
predominantly (although not exclusively) in endothelial cells (ECs), exerts proinflammatory, mitogenic, and profibrotic effects through the ET<sub>A</sub> receptor (ET<sub>A</sub>R).<sup>8,9</sup> At the level of glomerular micrcirculation, it has been reported that podocyte-specific activation of TGF-<b>B</b> signaling results in the release of ET-1 by visceral epithelial cells that act as paracrine stimulus for EC dysfunction through ET<sub>A</sub>R activation, setting in motion a vicious cycle that leads to podocyte depletion that eventuates in segmental glomerular damage.<sup>10</sup> This evidence prompted us to investigate the role of endothelial deregulation of ET-1/ET<sub>A</sub>R signaling in MWF rats, a well known model of progressive endothelial and podocyte loss.<sup>5,11</sup> To identify the cellular sources of ET-1, we performed multiple immunostaining and found that ET-1 protein was highly expressed by both ECs and podocytes, which were documented by costaining of Reca1 and α-actinin4, in kidneys of MWF compared with Wistar rats (Supplemental Figure 3A). Renal ET<sub>A</sub>R expression in the vascular endothelium of MWF rats was also increased in a time-dependent manner (Supplemental Figure 3B). Angiotensin II inhibition markedly reduced ET-1 renal expression to control levels, particularly in cortical interstitium (Supplemental Figure 3A), and normalized ET<sub>A</sub>R endothelial expression, which was indicated by reduced ET<sub>A</sub>R staining in Reca1-positive cells (Supplemental Figure 3B).

Both TGF-β2 and ET-1 promote the endothelial-to-mesenchymal transition,
which generates matrix-producing fibroblasts and/or myofibroblasts, while directly leading to EC loss.\textsuperscript{12–15} Although we cannot exclude the contribution of pericyte detachment from EC to scar-forming myofibroblasts, to assess the contribution of the endothelial-to-mesenchymal transition to vascular rarefaction, we evaluated the expression of the mesenchymal marker $\alpha$-smooth muscle actin ($\alpha$SMA) at the vascular level by means of colocalization with the Reca1 antigen. Glomerular and interstitial peritubular capillaries of MWF rats strongly expressed $\alpha$SMA, whereas only occasional staining was observed in Wistar rats. RAS blockade by ACEi and AIIRA significantly reduced $\alpha$SMA expression in kidney microvasculature of MWF rats to an equal extent (Figure 3, C and D). A similar mechanism seems to operate at the level of larger vessels to the extent that $\alpha$SMA staining progressively increased, reaching a peak at 60 weeks in MWF rats. RAS blockade did not simply lower $\alpha$SMA accumulation in large vessels but even normalized it in most animals (Figure 3E).

Collectively, these data indicate that inhibition of TGF-$\beta$ and ET-1/ET$_{A}$R signaling likely explains the beneficial effect of RAS blockade on the regeneration of kidney vasculature in advanced nephropathy. Because on RAS blockade, new vessel segment and capillary formation occurs, we further explored how the balance between apoptosis and proliferation at the EC level could account for vessel regeneration. Activated caspase3 was strongly up-regulated in Reca1-positive ECs in MWF rats at 50 weeks of age and even more at 60 weeks of age (Figure 4A). However, ECs were induced to proliferate, which was confirmed by the increased number of Ki67-positive cells in the renal vascular compartment, possibly to counterbalance the onset of the apoptotic events (Figure 4B). Angiotensin II inhibition significantly

\textbf{Figure 4.} RAS blockade reduces EC apoptosis, while increases proliferation, and restores Nrf2 expression. (A) Immunostaining for Reca1 (white) and caspase3 (red). Apoptotic ECs were evaluated by semiquantitative score (from zero to three in each field). (B) Immunostaining and quantification of Reca1 (white) and Ki67 (red) expression in untreated and lisinopril–or losartan–treated MWF rats. Nuclei were stained with DAPI (blue), and renal structures were stained with WGA (green). The enlarged details are shown in Insets in A and B. (C) Immunostaining for Nrf2 in formalin–fixed, paraffin–embedded kidney sections by immunoperoxidase technique. Nrf2 expression was evaluated by semiquantitative score (from zero to three in each field). Values are means±SDs (A and B, $n=3$; C, $n=4$). Scale bar, 50 $\mu$m. **$P<0.01$ versus MWF60; ***$P<0.01$ versus MWF50. DAPI, 4`,6-diamidin-2-fenilindolo LIS, lisinopril; LOS, losartan; Reca1, rat endothelial cell antigen1; WGA, wheat germ agglutinin.
reduced the number of apoptotic cells and sustained the compensating protective mechanism fostering EC proliferation, which could account for the important increase in the density and length of the microvessels observed by microCT (Figure 1, C and D) in treated MWF rats. NF-E2–related factor2 (Nrf2) has been recently recognized as a critical intracellular regulator of EC dynamics, governing angiogenic sprouting and vascular branching.16 Such pathways can be theoretically targeted therapeutically to counteract the effects of oxidative stress and TGF-β/Smad–mediated renal fibrosis.17–20 Finding here that RAS blockade rescues Nrf2 expression (as shown in Figure 4C), which was significantly decreased in untreated MWF rats, is consistent with this possibility.

In conclusion, our findings of vascular rarefaction in progressive renal disease in the MWF rat and vascular regeneration on RAS inhibition disclose a new paradigm for renal disease progression and offer room for therapeutic interventions. Vascular regeneration by angiotensin II antagonism, documented here for the first time, encompasses the entire vascular tree and is apparently accomplished by halting TGF-β– and ET-1–mediated damage. This phenomenon enhances the intrinsic reparative capability of the kidney by balancing regeneration and fibrosis and promoting angiogenesis, Nrf2–dependent vascular remodeling, and EC proliferation. These effects translate into improvement of tissue perfusion and filtration capacity and regression of kidney fibrosis. These results have obvious important implications for human medicine, showing effective improvement of an important clinical problem2 that has costs are becoming problematic, even for wealthier nations.

**CONCISE METHODS**

Animal Studies

The study design is graphically reported in Figure 1A, and more detailed information is reported in Supplemental Material. Briefly, one group of Wistar rats was studied at 30 weeks of age, and four groups of MWF rats were studied at 50 and 60 weeks of age with and without treatment with lisinopril or losartan. Kidney vasculature was investigated by microCT imaging with the use of a radio-opaque resin (Microfil; Flow Tech) injected in the kidney under general anesthesia (Supplemental Material). Imaging by microCT was obtained by three–dimensional numeric reconstruction using the NRecon software (Bruker-MicroCT), and quantification of vasculature components was performed using CTAlyser software (Bruker-MicroCT). Details on calculations and statistics of geometric parameters of kidney blood vessels are reported in Supplemental Material. Glomerular microcirculation was investigated by morphologic and quantitative SEM using a casting technique and imaging on a scanning electron microscope (Cross-Beam 1540EsB; Carl Zeiss). Morphometric analysis was performed on digital images to estimate GC dimensions as described in Supplemental Material.

**Gene Expression Analysis and Immunohistochemistry**

Total RNA was extracted with the Qiagen RNasy Mini Kit and reverse transcribed with the RT2 First-Strand Kit (Qiagen). The Rat Angiogenesis Pathway RT2 Profiler PCR Array (Qiagen) was used to determine the differentially expressed genes. Validation of Tgf-β2 and Et-1 gene expression was performed by quantitative RT-PCR. Reca1, αSMA, Ki67, caspase3, ETαR, ET-1, and α-actinin4 protein expression was evaluated by immunofluorescence experiments, whereas Nrf2 expression was detected by the immunoperoxidase technique, which is specified in Supplemental Material.

**Statistical Analyses**

Data are expressed as means ± SDs or medians and ranges as specified. Statistical analysis was performed using ANOVA (Prism 6.0; GraphPad Software Inc., San Diego, CA). Bonferroni post hoc analysis was adopted to estimate statistical significance of between groups’ comparisons. Statistical significance was defined as P<0.05.

**Study Approval**

Animal care and treatment were conducted in accordance with the institutional guidelines and compliance with national (DE 116, GU 18/2/1992, Circ. 8, and G.U 14/7/1994) and international laws and policies (Dir. 2010/65/EU and 9/22/2010). All animal studies were approved by the Institutional Animal Care and Use Committee of Istituto di Ricerche Farmacologiche Mario Negri.

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**DISCLOSURES**

None.

**REFERENCES**


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Supplementary Fig. S1. Renal histology of MWF rats untreated and treated with RAS inhibitors. (A) Representative images of PAS-stained renal sections showing glomerular and tubulointerstitial damage in 50 and 60 week-old MWF rats in respect to control Wistar rats. Treatment with lisinopril or losartan effectively ameliorated renal structure in respect to rats not receiving any treatment. Scale bars = 100μm. (B) Enlarged images showing glomerular lesions in detail. Scale bars = 50μm. (C) Histogram reports the incidence of glomerulosclerosis expressed as index in Wistar and MWF rats untreated and treated with RAS inhibitors. ** P<0.01 vs. MWF 50, *** P<0.01 vs. MWF 60.
Supplementary Fig. S2. Extensive representation of glomerular capillary lumen in Wistar rats and in MWF rats obtained by corrosion cast and SEM analysis.
Supplementary Fig. S3. Effect of lisinopril and losartan in reducing ET1 and ET_{A}R expression. (a) Representative images for triple immunostaining for ET1 (red), Reca1 (white) and the podocyte marker α-actinin4 (green), showing that ET1 is expressed by both endothelial cells and podocytes. On the bottom, lower magnification pictures shows that ET1 expression is increased in MWF rats and was reduced by lisinopril and losartan. Scale bar = 50μm. (b) Double immunostaining for Reca1 (green) and ET_{A}R (red). The expression of ET_{A}R on endothelial cells was evaluated by giving a semi-quantitative score from 0 to 3 in each field. Histogram shows that both treatments are effective in reducing the amount of ET_{A}R on endothelial cells. Scale bar = 50μm. Values are mean ±SD (n = 4). * P < 0.05 and ** P < 0.01 vs MWF 60.
ONLINE METHODS

Study design. Eight Wistar rats (Charles River S.p.A, Calco, Italy) and forty-one male MWF rats from our colony1 were used in this study. The group of Wistar rats (n=8) was studied at 50 weeks of age and used as normal control. MWF rats were divided into four groups. Group 1, MWF 50 (n=8) consisting of untreated animals, was studied at 50 weeks of age. Group 2, MWF 60 (n=13) was left untreated and followed from 50 to 60 weeks of age. Group 3, MWF+LIS 60 (n=13) and group 4, MWF+LOS 60 (n=7) were treated from 50 to 60 weeks of age with the ACE inhibitor lisinopril (80 mg/L in drinking water) or with the angiotensin II receptor antagonist losartan (100 mg/kg body weight in drinking water), respectively. Two additional groups of Wistar rats (n=4 in each group), of 30 weeks of age, have been used to investigate the effect of ACE inhibition on kidney perfusion by Microfil. Twenty-four hour urinary protein excretion was periodically measured during the observation period by Coomassie method using a Cobas Mira auto analyzer (Roche Diagnostic). All animals were housed in a temperature-controlled room regulated with a 12-hours dark/12-hours light cycle and allowed free access to standard diet containing 18% protein by weight and water.

MicroCT imaging of kidney vasculature. At sacrifice, rats were anesthetized with isofluorane. A PE50 catheter was placed in the thoracic aorta, and the vessel was ligated below and above renal arteries. The right kidney was removed and kidney specimens were obtained for further processing for PCR, renal histology and immunohistochemistry analyses. The vena cava was cut to allow free blood outflow and the left kidney was perfused with saline containing heparin (10 U.I./mL). A freshly mixed radio-opaque silicone polymer containing lead chromate (Microfil MV122, Flow Tech, Carver, MA, USA) was then infused, under a constant pressure of about 120
mmHg, until free drain was observed. Renal vein and artery were ligated, the kidney was left in situ for 20 min to allow Microfil polymerization and finally removed. After complete polymerization (about 2 hours) the kidney was immersed in 4% formaldehyde buffered with 0.1 M phosphate (pH 7.2) to complete fixation and left at 4°C. After three days, samples were imaged by microCT system (SkyScan 1076, Bruker-microCT, Kontich, Belgium). Scanning was performed using an aluminium filter (0.5 mm) with source voltage of 47 kV, current 200 mA, exposure time 1800 ms and rotational step of 0.4°. Volume images were reconstructed using a back-projection algorithm using NRecon software (Bruker-microCT) at pixel size of 9 µm with 256 gray levels.

Kidney segmentation was obtained by a semi-automatic procedure based on region growing algorithm using open source MITK software (www.mitk.org). Quantitative 3D structure analysis was then performed using CTAnalyser software (Bruker-microCT). After image binarization total kidney volume (TK_v) was computed applying the marching cubes algorithm. For vascular structure segmentation, images were thresholded above gray level 130. Total blood vessel volume (TV_v) was calculated on thresholded images with marching cubes algorithm. Vascular volume density (V_v) was calculated as the ratio between TV_v and TK_v. Vessels diameters along the vascular tree were computed using the "sphere-fitting" method reported by Hildebrand et al. This method allows the direct estimation of structure thickness without a model-assumption and this well applies for the analysis of objects with a complex structure as kidney vasculature. Being Ω ∈ R³ the set of all points of the spatial structure and p ∈ Ω an arbitrary point in this structure, local thickness τ(p) is defined as the diameter of the largest sphere which fulfils the conditions of enclosing the point p (which is not necessarily the center of the sphere) and of being entirely bounded within the solid surfaces according to the following equation
\[
\tau(p) = 2 \cdot \max(\{r \mid p \in \text{sp}(\vec{x}, r) \subseteq \Omega, \vec{x} \in \Omega\})
\]

where \(\text{sp}(\vec{x}, r)\) is the set of points contained in the sphere of center \(\vec{x}\) and radius \(r\).

The maximal local thickness is equivalent to the diameter of the largest sphere that completely fits inside the structure

\[
\tau_{\text{max}} = \max \left( \left\{ \tau(p) \mid p \in \Omega \right\} \right)
\]

The numerical method starts with a "skeletonisation" used to identify medial axes of all vascular structures, then the "sphere-fitting" local thickness measurement is applied to all the voxels lying along this axis. Blood vessels are then classified according to their diameters in three groups, microvessels with diameter lower than 80 µm, microvessels with diameter between 80 and 180 µm and large vessels with diameter over 180 µm. For each group of vessels, the mean diameter is computed and used to calculate the length of an equivalent vessel, having the same mean diameter and volume.

\textit{SEM and morphometric analysis}. Vascular corrosion casting was performed in selected animals from Wistar 50, MWF 50, MWF 60 and MWF+LIS 60 group (n=3 each) as described previously. After animal preparation described above, the left kidney was first perfused with heparinised saline followed by infusion of 2% paraformaldehyde in phosphate-buffered saline. Thereafter, the polyurethane resin PU4ii (vasQtec, Zurich, Switzerland, diluted with ethylmethylketone at 30% w/v with addition of a blue pigment, resin/hardener ratio, 6:1 by weight) was infused until complete perfusion of the kidney that uniformly stained in blue. The kidney was
excised and maintained at room temperature for 48 hours. After resin curing, soft
tissue was macerated in 7.5% KOH for 48 hours at 50°C and the resin cast was
thoroughly rinsed with water and freeze-dried.

Casted glomeruli were dissected from the cortex under a stereomicroscope,
mounted onto aluminum stubs on an adhesive film, and coated with 10 nm of gold in
a sputter-coater (Agar Scientific Ltd, Stansted, England) with a current of 10 mA for
60 s. Low and high power images of microvascular corrosion casts were taken with a
Cross-Beam 1540EsB scanning electron microscope (Carl Zeiss GmbH, Oberkochen,
Germany). Filament current was set to 2 kV and in-lens detector was used to
generate images of secondary electrons. For morphometric analysis images of 75
randomly selected casted glomeruli were acquired for each animal at fixed
magnification (703x).

Morphometric analysis was performed on digital images (1024x768 pixels) using
ImageJ software (NIH, Bethesda, MD, USA). To estimate mean glomerular volume
($V_G$), the outline of the glomerular tuft was manually traced on digital images, and its
area automatically computed. Under the hypothesis of spherical geometry, the radius
and $V_G$ of each glomerulus were then calculated from maximal glomerular area. To
quantify the regularity of the shape of the glomerular capillary tuft, the circularity
($C_{gc}$) of the maximal glomerular area ($A$) was calculated using a specific ImageJ
plugin,

$$C_{gc} = 4\pi \frac{A}{p^2}$$

where $p$ is the perimeter of the glomerular tuft. A circularity value of 1 indicates a
perfect circle while a value $\rightarrow 0$ indicates elongated shape.
Mean diameter of glomerular capillary segments was estimated using morphometric analysis. An orthogonal grid (7x10 lines, with distance between lines of 55 µm) was superimposed on 50 SEM images of individual glomerular casts. For each image, the mean diameter of capillary segments was estimated using the harmonic mean of the grid line intersecting the capillary segments. We estimated the length of the glomerular capillaries in individual capillary tufts imaged by SEM on the basis of the resin volume (volume of the capillary lumen) and the mean capillary segment area calculated from mean capillary diameter. We calculated the length of single glomerular capillary as the ratio between lumen volume and mean capillary lumen area. To estimate the effective volume occupied by the resin (corresponding to capillary lumen volume) within the capillary tuft we obtained images of cross sections of the glomerular casts using a focused ion beam (FIB) cut performed on a SEM-FIB electron microscope (Cross-Beam 1540EsB, Carl Zeiss GmbH, Oberkochen, Germany). Digitized images were then processed for morphometric analysis to obtain, by point counting (superimposing a 20 x 29 orthogonal grid), the ratio between resin volume and total capillary tuft volume, assuming volume density equal to area density.

**PCR Array.** For real-time PCR array, total RNA was extracted from rat kidney specimens using Qiagen RNeasy Mini kit. RNA extracted from three different animals for each group was pooled and 1 µg used for the first-strand cDNA synthesis using the RT² First Strand Kit (Invitrogen). The Rat Angiogenesis Pathway RT² Profiler PCR Array (PARN-024Z; SABiosciences, Qiagen) was used to determine the differentially expressed genes. Data analysis was performed using the online SA bioscience software. The boundary (fold regulation cut off) was set to 2-fold change between the different groups.
Quantitative Real-Time PCR (qRT-PCR). Total RNA was extracted from kidney of each rat MWF or Wistar using Trizol reagent and contaminating genomic DNA was removed by RNase-free DNase (Promega) for 1 hour at 37°C. The first-strand cDNA (2 µg) was produced using SuperScript VILO cDNA Synthesis Kit (Life Technologies) following the manufacturer’s procedure. No enzyme was added for reverse transcriptase-negative controls (RT-). Amplification was performed on ViiA7 Real Time PCR System with SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer’s protocol. After an initial hold of 2 min at 50°C and 10 min at 95°C, the samples were cycled 40 times at 95°C for 15 s and 60°C for 60 s to reach the plateau. The comparative Ct method normalizes the number of target gene copies to the housekeeping gene as Gapdh (ΔCt). Gene expression was then evaluated by the quantification of cDNA corresponding with the target gene relative to a calibrator sample (Wistar rats) serving as physiologic reference (ΔΔCt). On the basis of exponential amplification of target gene as well as calibrator, the amount of amplified molecules at the threshold cycle is given by: $2^{-\Delta \Delta Ct}$. The following primers (300 nM) were used: rat TGF-β2 sense 5’-TAAAATCGACATGCCGTCCA-3’, antisense 5’-GGATGGCATCAAGGTACCCAC-3’; rat ET-1 sense 5’- CTGTTTGTTGCTTTCCAAGGA-3’, antisense 5’-CTCTGCTCCCAAGACAGCTGT-3’; GAPDH sense 5’-TCATCCCTGCATCCACTGGT-3’, antisense 5’-CTGGGATGACCTTGCCCAC-3’.

Renal histology. Kidney specimens were fixed in Duboscq-Brazil and embedded in paraffin. Three-micron sections were stained with periodic acid-Schiff (PAS) reagent and the incidence and extent of glomerular and tubular structural lesions were estimated at light microscopy (ApoTome Axio Imager Z2, Zeiss, Jena, Germany). An average of 35 glomeruli was examined for each animal. Each glomerulus was scored according to the extent of sclerotic changes consisting of matrix deposition, capillary
occlusion and capillary tuft adhesion to Bowman's capsule. Score was assigned to 0 in the absence of sclerosis, to 1 for changes affecting less than 25% of the glomerular area, to 2 and 3 for lesions affecting 25–50 and 50–75% of the tuft, respectively, and to 4 for lesion exceeding 75% of the tuft. The average GS index in each animal was then calculated as weighted mean. Tubular structural changes were evaluated by semiquantitative scores. Tubular atrophy, interstitial fibrosis and inflammation and luminal casts were graded from 0 to 3+ (0=no changes; 1+=changes affecting less than 25% of the sample; 2+=changes affecting 25 to 50% of the sample; 3+=changes affecting more than 50% of the sample). At least 10 fields per kidney slice were examined for histological scores at low-power magnification. All tissue sections were analyzed by the same pathologist in a single-blinded manner.

**Immunofluorescence experiments.** PLP or Acetone-fixed cryosections were post-fixed with cold acetone, treated with 1% bovine serum albumin (BSA) to block nonspecific sites and incubated with the following antibodies: mouse anti-Reca1 (1:50, AbDSerotec, Kidlingdon, Oxford, United Kingdom), Cy3-conjugated mouse anti-αSMA (1:100, Sigma Aldrich, St Louis, MO, USA), rabbit anti-ki67 (1:200, AbCam, Cambridge, UK), rabbit anti-caspase3 (1:100, Cell Signaling, Beverly, MA, USA), rabbit anti-ET₁R (1:200, Alomone Labs, Jerusalem, Israel), goat anti-ET1 (1:100, Santa Cruz), rabbit anti-α-actinin4 (1:150, OriGene Technologies, Rockville, MD), followed by the specific FITC, Cy3 or Cy5-conjugated secondary antibodies. Nuclei were stained with DAPI, and renal structures with FITC wheat germ agglutinin (WGA). Negative controls were obtained by omitting primary antibodies on adjacent sections. Fluorescence was examined by confocal laser microscopy (LS 510Meta, Zeiss). Capillary volume density (Vv) was quantified as Reca1–positive vessels in 20 tubulointerstitial fields and 20 glomeruli per sample (n=4 for each group) randomly

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acquired. By using the analysis software ImageJ 1.40g, digitized images were binarized using a threshold for areas of Reca1 staining, and the values were expressed as percentage of area occupied by Reca1 on total area of the acquired field. Reca1-αSMA co-staining was evaluated in an average of 10 glomeruli and 10 high power fields of interstitial area (HPF, x40) in each kidney sample. Merge signal was quantified using an appropriate software (Axio Vision, Apotome Axio Imager Z2, Zeiss) and expressed as percentage of Reca1/αSMA-positive area on total Reca1-positive area per field. Interstitial fibrotic vessels were identified as large vessels with an abnormal αSMA accumulation, and quantification was expressed as percentage on the total number of Reca1+ large vessels. Endothelial cell proliferation was quantified as the number of Reca1+Ki67+ cells in each field (40X). Reca1-caspase3 and Reca1-ETαR expression were evaluated as semi-quantitative score of the merge positive area per HPF (0: no signal, 1: occasional, 2: mild, 3: diffuse).

**Immunoperoxidase experiment.** Formalin fixed, paraffin embedded kidney sections (3 μm) were deparaffinized, hydrated and incubated for 30 minutes with 0.3 % H₂O₂ in methanol to quench endogenous peroxidase. Antigen retrieval was performed by boiling sections using microwave (twice for 5 min in citrate buffer 10 mM, pH 6.0 at operating frequency of 2450 MHz and 600 W power output). Sections were blocked with 1% bovine serum albumin (BSA), and subsequently incubated overnight with rabbit anti-NRF2 antibody (diluted 1:50, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). After incubation with specie-specific biotinylated secondary antibody and diaminobenzidine (DAB, Merck, Darmstadt, Germany) substrate solution, slides were counterstained with hematoxylin, dehydrated in graded alcohols, mounted with coverslips and observed by light microscopy (Primo Star, Zeiss, Jena, Germany). Negative controls were obtained by omitting the primary antibody on
adjacent sections. Nrf2 signal was graded on a scale from 0 to 3 on the basis of the number of positive structures in each field (0: no signal, 1: from 1 to 5, 2: from 6 to 10, 3: more than 10).
REFERENCES


Supplementary Table S1: Structural changes of kidney tissue in Wistar and MWF rats.

<table>
<thead>
<tr>
<th></th>
<th>Wistar 50</th>
<th>MWF 50</th>
<th>MWF 60</th>
<th>MWF+LIS 60</th>
<th>MWF+LOS 60</th>
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<tbody>
<tr>
<td>Tubular atrophy</td>
<td>0.25±0.26</td>
<td>2.22±0.26</td>
<td>2.50±0.43</td>
<td>1.17±0.25</td>
<td>1.46±0.14</td>
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<tr>
<td>(score 0-3)</td>
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<td><strong>°</strong></td>
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<tr>
<td>Interstitial fibrosis</td>
<td>0.04±0.14</td>
<td>2.28±0.26</td>
<td>2.44±0.46</td>
<td>1.04±0.50</td>
<td>1.50±0.56</td>
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<td><strong>°°</strong></td>
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<tr>
<td>Interstitial Inflammation</td>
<td>0.08±0.19</td>
<td>2.33±0.25</td>
<td>2.61±0.33</td>
<td>0.92±0.42</td>
<td>1.38±0.53</td>
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<tr>
<td>Luminal casts</td>
<td>0.08±0.19</td>
<td>1.50±0.50</td>
<td>2.11±0.70</td>
<td>0.71±0.45</td>
<td>1.08±0.63</td>
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</tbody>
</table>

° P<0.05 vs MWF 50
** P<0.01 vs MWF 50
*** P<0.01 vs MWF 60