Role of Pressure in Angiotensin II-Induced Renal Injury
Chronic Servo-Control of Renal Perfusion Pressure in Rats

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Abstract—Renal perfusion pressure was servo-controlled chronically in rats to quantify the relative contribution of elevated arterial pressure versus angiotensin II (Ang II) on the induction of renal injury in Ang II-induced hypertension. Sprague-Dawley rats fed a 4% salt diet were administered Ang II for 14 days (25 ng/kg per minute IV; saline only for sham rats), and the renal perfusion pressure to the left kidney was continuously servo-controlled to maintain a normal pressure in that kidney throughout the period of hypertension. An aortic occluder was implanted around the aorta between the two renal arteries and carotid and femoral arterial pressure were measured continuously throughout the experiment to determine uncontrolled and controlled renal perfusion pressure, respectively. Renal perfusion pressure of uncontrolled, controlled, and sham kidneys over the period of Ang II or saline infusion averaged 152.6±7.0, 117.4±3.5, and 110.7±2.2 mm Hg, respectively. The high-pressure uncontrolled kidneys exhibited tubular necrosis and interstitial fibrosis, especially prominent in the outer medullary region. Regional glomerular sclerosis and interlobular artery injury were also pronounced. Controlled kidneys were significantly protected from interlobular artery injury, juxtamedullary glomeruli injury, tubular necrosis, and interstitial fibrosis as determined by comparing the level of injury. Glomerular injury was not prevented in the outer cortex. Transforming growth factor (TGF)-β and active NF-κB proteins determined by immunohistochemistry were colocalized in the uncontrolled kidney in regions of interstitial fibrosis. We conclude that the preferential juxtamedullary injury found in Ang II hypertension is largely induced by pressure and is probably mediated through the TGF-β and NF-κB pathway. (Hypertension. 2004;43:752-759.)

Key Words: angiotensin II ■ renal injury ■ kidney ■ renal perfusion pressure

Epidemiological studies show subjects with hypertension are at great risk for the development of end-stage renal disease.1 Yet it is also recognized that the association between end-stage renal disease and hypertension varies widely in human populations2 and in various primary and secondary forms of experimental hypertension.3 Antihypertensive agents afford protection in some individuals with hypertension, whereas in others they do not.4 The morphological damage of the kidneys from rats with sustained hypertension therefore appears to be dependent on complex interactions between the blood pressure and other factors that predispose the kidneys to damage including genes of susceptibility and the exposure of the kidneys to various paracrine and endocrine factors. That being said, it is evident from the literature that the role of blood pressure per se in renal injury remains poorly understood and is a continuing source of debate.

The present study was performed to more precisely determine the role of blood pressure in the renal morphological damage that is observed in a very specific form of hypertension, that induced by angiotensin II (Ang II). Chronic subcutaneous infusion of Ang II (200 ng/min) that produced substantial hypertension has been shown to induce tubular atrophy and dilation, cast formation, interstitial monocytic infiltrate, and mild interstitial fibrosis in rats.5 Double transgenic rat models of hypertension in rats producing excess Ang II exhibit prominent renal injury.6 These studies, however, have not enabled investigators to determine the extent to which the renal damage was a consequence of the blood pressure or the direct effects of Ang II. Because Ang II has mitogenic actions, it has been proposed that many of the observed morphological changes could be a direct consequence of Ang II rather than the elevated blood pressure.7 Studies using the 2-kidney 1-clip Goldblatt model of hypertension in which the nonclipped contralateral kidney is compared with the clipped or "pressure-protected" kidney have provided some insights into the question showing that the kidney exposed to high renal perfusion pressure (RPP) exhibits glomerular, tubular, and vascular injury.8 Nevertheless, the morphology of the clipped kidney is difficult to assess because the clipped kidney is exposed to an undefined period of ischemia after placement of the renal artery clip.

The present study was therefore designed to quantify with considerable precision the relative contribution of pressure versus Ang II on the renal injury in the Ang II-induced model of hypertension in Sprague-Dawley rats. This was assessed by the application of techniques that enabled the continuous...
servo-control of arterial pressure at normal levels to the left kidney while the right kidney was exposed to hypertension for a period of 2 weeks.

Methods

Experimental Animals
Studies used male Sprague-Dawley rats weighing 250 to 350 g (Harlan, Madison, Wis) provided a standard pellet diet (Purina Mills, St. Louis, Mo) and water ad libitum. The chronic studies were performed in the Animal Resource Center of the Medical College of Wisconsin.

Surgical Preparation
Rats were anesthetized with ketamine (100 mg/kg, IM) and acepromazine (2 mg/kg, IM) for implantation of in-dwelling arterial (right carotid artery and left femoral artery) and venous (left femoral vein) catheters as described previously.9 An inflatable silastic vascular occluder (1.5-mm lumen diameter, 2.5-mm width; In Vivo Metric) was implanted around the aorta between the right and left renal artery branches through a mid-sagittal abdominal incision then attached to an 80-cm length of flexible Tygon tubing (0.76-mm inner and 2.29-mm outer diameter) that was exteriorized at the back of the neck. Average time for surgery was 75 minutes, and rats were recovered for 7 days before the study.

Hemodynamic Measurements and Servo-Control of Renal Perfusion Pressure
A servo-controlled turntable (Rodent workstation with Ratum system; Bioanalytical Systems, West Lafayette, Ind) was used that enabled free movement of the rat throughout the study. Rats were conditioned to the turntable for 3 to 4 days before undergoing any surgical procedures. This was accomplished by the automatic counter rotation of the circular cage in a direction opposite to the rat movement. A continuous infusion of saline or drug was delivered at a rate of 6.9 μL/min through the venous catheter. Femoral and carotid arterial catheter patency was maintained throughout the study by the slow infusion (1.7 μL/min) of 30 U/mL of heparin throughout the study. A 1-way check-valve was inserted between the arterial catheters and the infusion pump to prevent back diffusion of blood into the arterial lines during the continuous 24-hour recordings of arterial pressure. The pressure signal from the femoral arterial catheter, reflecting RPP of the left kidney, was the input to the servo-control unit that initiated the inflation of the occluder cuff. The response speed was adjusted to maintain mean arterial pressure constant within 1-minute time periods at a level ±10 mm Hg around the average 24-hour control pressure determined during the 3 control days before initiation of the Ang II infusion.

Experimental Design
Servo-Controlled Ang II-Infused Rats
Four days after surgery, the food was switched to a purified 45-mg small pellet diet containing 4% salt (Research Diets) as necessitated by the caging system. On day 7, daily recordings of mean arterial pressure (MAP) began and continued for 3 days, at which time the intravenous saline infusion was switched to Ang II (25 ng/kg per minute; 6.9 μL/min) and continued for 14 days. The servo-control of RPP was initiated on the morning after the initial increase in pressure in response to Ang II. A 0.6-ml blood sample was drawn for plasma renin activity on the final control day and on the final day of Ang II infusion.

Sham-Operated Saline Infused Rats
Surgical implantation of the catheters, including the aortic occluder was identical in this group of rats. In this group, saline was infused continuously for 14 days, 4% salt diet was fed, and the occluder was not inflated.

Histology Preparation and Measurements
Rats were euthanized by excess sodium pentobarbital (100 mg/kg) at the end of the study and the kidneys and aorta were removed, immersion-fixed in 10% neutral buffered formalin, and paraffin-embedded. Sections were stained with Gomori trichrome stain to highlight the fibrotic tissue. Interlobular artery injury was determined by 2 methods. First, 20 random interlobular arteries were graded from 0 to 4 as determined by obstruction of the vessel lumen (0 is normal; 1, hypercellularity of intima or destruction of internal elastic layer with <25% of inner lumen involved in stenosis; 2, 25% to 50% involved; 3, 50% to 75% involved; 4, 75% to 100% involved). Second, the wall thickness was determined by tracing the inner and outer circumference with image analysis software and determining the ratio of the area of the inner lumen to the area determined using the outer circumference of the vessel. The size of the vessel was normalized by calculating the ratio of median wall area dimension to the area of outer circumference. Glomerulosclerosis and mesangial matrix expansion were scored on a scale of 0 to 4 as described by Raj et al.11 Each section was scored twice (blindly) and the average was used for analysis. Interstitial fibrosis was determined by immunostaining with antibodies for α-smooth muscle actin (SMA) (Dako Cytomation) and fibronectin (Santa Cruz Biotechnology). Adjoining serial sections were also immunostained with transforming growth factor (TGF)-β1 (Santa Cruz Biotechnol-ogy) and NF-κB (activated p65 subunit; Chemicon) and were stained with picro sirius red to detect collagen types I and III.12 SMA, fibronectin, and TGF-β1 staining were detected with an Envision/HRP detection kit (Dako Cytomation). NF-κB was detected with LSAB+ detection kit (Dako Cytomation). A robotic DAKO autostainer (S3400; Dako Cytomation) was used for all immunostaining so that all samples were stained under the same conditions in parallel. The percentage of the SMA and fibronectin-positive region was determined in 20 randomly chosen frames at 100× magnification captured by Nikon E400 microscope (Fryer Co) equipped with a Spot Insight color CCD camera (Diagnostic Instruments), which included nearly the entire outer medulla and quantified using Metamorph image analysis software (version 4.6; Universal Imaging Systems Corp). Adjacent serial sections were stained with hematox- ylin and eosin to quantify cast formation as an index of tubular dysfunction. Eosin has high autofluorescence, so a threshold well above background could be established, and the highly fluorescent cast region could be determined by 490 nm excitation and 535/40 nm emission using a Nikon E600 fluorescent microscope (Fryer Co) equipped with a Micromax cooled CCD camera (Princeton Instruments). The percentage of the cast region was acquired in 20 randomly chosen frames per outer medulla of kidney at 100× magnification and also quantified by using Metamorph image analysis software.

The contribution to renal injury of arterial pressure versus the direct effects of Ang II was determined by comparing the level of injury (assessed by image analysis as described above) of the servo-controlled left kidney to those of the uncontrolled right kidney. The calculation was made using the following equations:

\[
\text{Percentage of pressure induced injury} = \left[ \frac{(U - C)}{(U - S)} \right] \times 100(\%)
\]

\[
\text{Percentage of Ang II induced injury} = \left[ \frac{(C - S)}{(U - S)} \right] \times 100(\%)
\]

where U, C, and S are the injury score values of uncontrolled (U), controlled (C), and average of 5 sham kidneys (S).

Quantification of the levels of injury was enhanced by using an autostainer that could simultaneously stain the 34 slides (including the negative controls) under identical conditions. When combined with strictly controlled software settings for image quantification, the variance of results could be minimized.

Statistical Analysis
Data are expressed as mean±SE. Daily blood pressure changes were determined by 1-way ANOVA for repeated measures followed by a Bonferroni test to determine differences between the baseline period and the 14 days of Ang II infusion. Paired t test was used to compare the uncontrolled to the controlled kidney. Unpaired t test was used to
compare the sham to the controlled kidney. Pearson correlation and linear regression analysis were performed to assess the relationship between RPP and renal indices of renal injury. The level of significance for all analyses was $P<0.05$.

## Results

### Hemodynamic and Biochemical Measurements in the Servo-Controlled Rat

Arterial pressure measured from the carotid artery was used as an index of right (uncontrolled) kidney perfusion pressure while the pressure measured from the femoral artery was used as an index of left (controlled) kidney perfusion pressure. A typical pressure profile plotted as 3-minute averages is shown in Figure 1A. Left RPP was controlled to a level equivalent to that of the average baseline pressure. It can be seen in this recording that diurnal variations of almost 50 mm Hg became prominent and recurrent during the Ang II infusion period. The average fluctuations of RPP occurring over the 3-minute averages were less in the servo-controlled kidney than that in uncontrolled kidney. However, we can only speculate about the extent to which the higher frequency ($\approx$ 1 minute) or lower frequency ($\geq$ 3 minutes) fluctuations may have influenced the observed changes in renal pathology. The average RPP of the controlled and uncontrolled kidneys of the 6 rats studied is summarized in Figure 1B. RPP increased from a control pressure of 119.8 $\pm$ 1.8 mm Hg to 136.4 $\pm$ 4.6 mm Hg during the first day of Ang II infusion just before the beginning of the servo-control of pressure. The average 24-hour RPP of the servo-controlled left kidney remained within $\pm$ 2 mm Hg of baseline pressure values in all studies and increased from an average baseline pressure of 119.4 $\pm$ 1.0 mm Hg to 152.2 $\pm$ 7.0 mm Hg for the uncontrolled kidney during the Ang II infusion period. By the day 8 of servo-control, RPP above the servo-controlled occluder reached an average plateau of 160.1 $\pm$ 9.5 mm Hg. RPP of the servo-controlled kidney remained unchanged from the initial baseline pressure of 115.0 $\pm$ 1.2 mm Hg to an average value of 117.0 $\pm$ 3.0 mm Hg over the servo-controlled period of Ang II infusion (Figure 1B). RPP of saline-infused, sham-operated rats averaged 109.6 $\pm$ 1.7 mm Hg ($n=5$) during the baseline period and did not change significantly ($P=0.70$) during the 14 days of study (110.7 $\pm$ 2.2 mm Hg). The average RPP of the 14-day experimental period was not statistically different between servo-controlled (117.0 $\pm$ 3.0 mm Hg) and sham control rats (110.5 $\pm$ 2.1 mm Hg).

The average aortic pulse pressure increased from 42.4 $\pm$ 0.7 mm Hg to 52.5 $\pm$ 1.8 mm Hg ($P<0.05$, $n=6$) above the aortic occluder as determined at days 12 to 14, while pulse pressure of the servo-controlled portion of the aorta decreased from 52.5 $\pm$ 1.8 mm Hg to 29.1 $\pm$ 4.3 mm Hg ($P<0.05$, $n=6$). Plasma renin activity was significantly suppressed during the Ang II-infused servo-control period averaging 0.7 $\pm$ 0.2 ng Ang I/mL per hour ($n=6$) compared with the baseline level of 1.6 $\pm$ 0.5 ng Ang I/mL per hour ($n=6$), indicating that renal underperfusion did not occur in the servo-controlled kidney.

![Figure 1](https://hyper.ahajournals.org/)

**Figure 1.** A, RPP is plotted at 3-minute intervals for a representative rat servo-controlled for 14 days. Both the pressure from the uncontrolled kidney (measured from the carotid artery) and from the controlled kidney (measured from the femoral artery) are shown. B, Daily average of RPP is plotted for uncontrolled ($\square; n=6$), controlled ($\bullet; n=6$), and sham ($\Delta; n=6$) kidneys. $^*P<0.05$ significantly increased from baseline period.

![Figure 2](https://hyper.ahajournals.org/)

**Figure 2.** Gomori trichrome staining of uncontrolled, controlled, and sham kidneys. Left panel, Glomeruli sclerosis and mesangial proliferation in regional juxtamedullary glomeruli observed in uncontrolled and controlled kidney. Right panel, Tubular necrosis with protein cast and interstitial fibrosis observed in outer medullary region of uncontrolled kidney and to a lesser extent in controlled kidney. Sham kidneys from the same regions were prepared for comparison.
technique for the juxtamedullary and outer medullary region. A more specific staining stain indicated the general pattern of damage in the juxtamedullary artery stenosis, as illustrated in Figure 2. The trichrome stain indicated the general pattern of damage in the juxtamedullary (left panel) and outer medullary (right panel) interstitial space in uncontrolled kidney, and to a lesser extent in controlled kidney, whereas those regions in the sham kidney had positive stained area only around the vessel wall.

**Histological Comparisons of the Uncontrolled, Controlled, and Sham-Operated Kidneys**

Uncontrolled kidneys exposed to the elevated arterial pressure were found to exhibit considerable tubular necrosis with protein casts, prominent interstitial fibrosis in the outer medullary region, regional glomerular sclerosis, and interlobular artery stenosis, as illustrated in Figure 2. The trichrome stain indicated the general pattern of damage in the juxtamedullary and outer medullary region. A more specific staining technique for α-SMA and fibronectin was applied to better define the more specific patterns of fibrosis as shown in Figure 3. Taken together, these 2 staining methods allowed for the quantitation of the renal damage in the uncontrolled and controlled kidneys as well as the sham kidney. Although a relatively small degree of interstitial fibrosis and tubular necrosis was present in the cortex of the uncontrolled kidney, this was quite focal compared with that seen in the medulla, and random samplings could not provide a reasonable quantification of the injury. The baseline immunostaining signal from the larger vessels in the cortex also precluded quantification in this region. The average differences in injury between uncontrolled and servo-controlled kidneys summarized were considered to represent the pressure-dependent effects because circulating levels of Ang II were the same in both kidneys. The differences in injury between servo-controlled kidneys and sham kidneys were considered to represent the effects of circulating Ang II, although it is a statistically less powerful comparison because these differences represented differences between rats rather than differences between the kidneys of the same rats.

**Glomerular Injury**

Approximately 10% to 15% of the glomeruli of kidneys exposed to the hypertension exhibited some degree of abnormality based on scores >1.0 as determined in a sampling of 50 glomeruli from each kidney. The total amount of glomerular injury in any region was not great in either the servo-controlled or uncontrolled kidneys (outer cortical glomeruli score averaging 1.0±0.1 and the juxtamedullary glomeruli averaging 1.1±0.1). However, when those glomeruli with a score of ≥2 were compared for the relative degree of injury, the juxtamedullary glomeruli were significantly more sclerosed in the uncontrolled kidney than in the controlled. As illustrated in Figure 4A, no significant differences were observed between the superficial glomeruli between the controlled and uncontrolled kidneys from servo-controlled rats. In contrast, kidneys protected from elevated pressures by servo-control exhibited a significant reduction in the number of injured juxtamedullary glomeruli when compared with the uncontrolled kidneys (4%±2% versus 16%±6%). Given the relatively mild degrees of glomerular injuries in this model, no attempt was made to separate between mesangial expansion and glomerulosclerosis. Both the servo-controlled and uncontrolled kidneys exhibited significantly (P<0.05) greater juxtamedullary glomerular injury than sham kidneys, where there was no apparent glomerular injury (n=5). The contribution (expressed as percent contribution to injury as calculated by the equation given in the Methods section) of arterial pressure to the outer cortical glomerular injury was minimal (9%±6%) compared with that attributable to Ang II that averaged 91%±6%, indicating that Ang II was the dominant cause of injury of the glomeruli in the outer cortical region. The contribution of arterial pressure to the juxtamedullary glomerular injury was 85%±8%, whereas that of Ang II was 15%±8%, indicating that pressure is the dominant cause of renal injury in the juxtamedullary region. The injury of the juxtamedullary glomeruli was significantly correlated with the average RPP of the Ang II-infused period (r=0.61, P<0.05), but not that of the outer cortical glomeruli (r=0.21, P=0.41) between pressures ranging between 106 to 170 mm Hg.

**Tubular Necrosis**

The percentage of the outer medullary region exhibiting protein cast was used as an index of tubular necrosis based on a modification of a method described previously. Protein cast staining (Figure 2) was found largely in thick ascending limbs and cast formation of the servo-controlled kidneys (0.3%±0.1%, n=6) was significantly (P<0.05) less than that of the uncontrolled (1.0%±0.2%, n=6) kidney. Cast staining in the servo-controlled kidneys was not significantly different than that of the sham kidneys (n=5; P=0.07), as shown in Figure 4B. Using these data, the calculated contribution of arterial pressure to tubular injury was 77%±7%, whereas the injury caused by Ang II was 23%±7%. A significant corre-
lation was observed between RPP and tubular necrosis ($r=0.74$, $P<0.05$) between pressures of 106 to 170 mm Hg.

**Preglomerular Vascular Injury**

Based on the 0 to 4 scoring method, the injury score of the uncontrolled kidneys averaged only $1.3\pm0.2$ (n=6 kidneys), so only the interlobular vessels exhibiting injury scores of 2 (25% to 50% obstruction) or greater were compared. As shown in Figure 4C, the scores of injured interlobular arteries of servo-controlled kidneys (7%±2%, n=6) were significantly ($P<0.05$) less than those of the uncontrolled (37%±7%, n=6) kidneys. Arterial pressure contributed 81%±7% to the injury of uncontrolled kidneys, whereas Ang II contributed 19%±7%. Similar results were obtained when comparisons were made using the vessel wall thickness in 20 randomly captured images of interlobular arteries (expressed as the ratio of the area of the inner lumen to the area determined using the outer circumference of the vessel). Specifically, wall thickness ratio of the interlobular arteries of uncontrolled kidneys (0.93±0.01, n=6) was significantly ($P<0.05$) greater than that of the servo-controlled (0.85±0.03, n=6) kidneys. The wall thickness ratio of the interlobular arteries of the uncontrolled kidneys, however, was not significantly ($P<0.05$) greater than those of the sham kidneys (0.82±0.02, n=5). It was estimated that arterial pressure contributed 63%±13% to the injury of uncontrolled kidneys, whereas Ang II contributed 37%±13%. A significant correlation was observed between RPP and injury of the interlobular artery, as determined by both the injury score ($r=0.86$, $P<0.05$) and the wall thickness ($r=0.53$, $P<0.05$), with pressures ranging from 106 to 170 mm Hg.

**Interstitial Fibrosis**

The percentage of tissue that stained positive for α-SMA and fibronectin in the outer medullary region is used as the index of interstitial fibrosis. As shown in Figure 4D, servo-controlled kidneys exhibited significantly less ($P<0.05$) interstitial fibrosis as represented by SMA (1.4%±0.1%; n=6) and fibronectin (7.6%±0.9%; n=6) than the uncontrolled kidneys (SMA 4.0%±0.3%; n=6; and fibronectin 17.0±2.9%, n=6). However, the servo-controlled kidney exhibited greater levels ($P<0.05$) of SMA and fibronectin compared with those of sham kidneys, averaging 0.6%±0.1% (SMA, n=5) and 1.7%±0.3% (fibronectin, n=5), respectively. The contribution of RPP involved in this injury as determined from SMA immunohistochemistry averaged 78%±2%, and those of fibronectin averaged 59%±6%. The injury attributable to the effects of Ang II alone averaged 22%±2% based on SMA and 41%±6% based on fibronectin staining. A significant correlation was observed between RPP and injury of interstitial fibrosis as determined by SMA ($r=0.90$, $P<0.05$) and fibronectin ($r=0.82$, $P<0.05$) immunostaining between pressures of 106 and 170 mm Hg. The data using either technique therefore indicate that the elevation of pressure was the dominant cause of interstitial fibrosis.

The signal transduction pathway involved in the observed renal fibrosis was explored by immunostaining with TGF-β1 and NF-κB (activated p65 subunit) antibodies applied to adjoining serial sections obtained from the same kidneys. These sections were also stained with picro sirius red for types I and III collagen. Although difficult to precisely quantify, as shown in Figure 5 both collagen type I and III,
TGF-β1, and activated NF-κB (shown as increased number of dark filled cell nuclei in fibrosis) appeared to be overexpressed in the same region of the outer medulla of the kidneys exposed to the elevated arterial pressure, as was observed for the overexpression of SMA and fibronectin shown in Figure 3 and Figure 4D. Taken together, these data indicate that this signaling pathway was involved in induction of pressure-induced fibrosis.

Discussion

In the present study, the application of servo-control techniques in instrumented unanesthetized rats enabled the independent control of RPP of the left and right kidneys that were exposed to the same chronically elevated circulating levels of Ang II. The results represent the first study in which RPP was controlled in rats for periods extending beyond 2 days.13

It is evident that during the initial several weeks of Ang II-induced hypertension, renal interstitial fibrosis and tubular injury had already begun to occur in the outer medulla of the kidney, and that ~70% to 75% of these changes were driven by a pressure-dependent mechanism. The renal cortex appeared to be relatively well protected from pressure-induced injury during this period. These differential effects between the cortex and medulla could be expected for several reasons. First, it is well known that the renal cortical blood flow exhibits a high degree of autoregulation,14 as does glomerular filtration, whereby the constriction of the afferent arterioles would protect the glomeruli from elevations of RPP. Second, it is recognized that the medullary blood flow of rats is not well autoregulated15,16 and that this diminished autoregulatory capacity enables increases of RPP to be transmitted to the vasa recta of the renal medullary circulation.15,16 These features of the renal medulla could make this region of the kidney more vulnerable to the injury of hypertension than the cortex, as we17 and others18 have speculated. It has been found that in hypertensive Dahl salt-sensitive rats, interstitial fibrosis and capillary injury occur first in the outer medulla.19 Consistent with these observations, it has been shown that hypertensive Dahl S rats exhibit more extensive renal damage and have diminished autoregulation of renal blood flow when compared with spontaneously hypertensive rats with the same level of blood pressure.20

Although a number of pathways could be triggered when elevated levels of arterial pressure are transmitted to the vasa recta of the outer medulla, there is reason to believe that the damage is related to increased levels of oxidative stress. Renal damage could be initiated by pressure or flow-induced endothelial cell dysfunction resulting in a reduced production of nitric oxide (NO).19,21 Reduction of NO production in the vasa recta microvessels that supply blood to the renal medulla has been shown to greatly enhance the vasoconstrictor effects of Ang II in this region and reduce tissue PO2 levels.22 Reduction of tissue NO in the renal medulla has also been shown to result in increased levels of oxygen free radicals,23 thus initiating oxidative stress. Because the renal medulla receives only ~5% of the total renal blood flow and the interstitial PO2 levels are normally only ~40 mm Hg in the outer medulla,17 increases in oxygen use in this region require increases of blood flow for adequate delivery of oxygen. Both reduction of medullary NO concentrations9 and increased levels of reactive oxygen species (O2- and H2O2) have been shown to reduce medullary blood flow and sodium excretion.24,25 This is especially relevant regarding the metabolic
needs of the medullary thick ascending limbs of Henle (mTAL) that are metabolically very active and mitochondria-rich.\textsuperscript{23,24} We have recently shown that Ang II increases NO release from mTAL\textsuperscript{26} and that this NO can diffuse to the surrounding vasa recta pericytes.\textsuperscript{26} Furthermore, we have found that increased production of superoxide radicals in the epithelial cells of the mTAL attenuates the release of NO into the interstitial space.\textsuperscript{23} As ischemia develops in the renal medulla, we hypothesize that even greater levels of oxidative stress would occur,\textsuperscript{23} resulting in further attenuation of NO diffusion. This positive feedback response would be expected to result in even greater levels of oxidative stress and lead to the tubular injury and fibrosis. As outlined in Figure 6, such a "tubulointerstitial vicious cycle" in the outer medullary region could explain why these injuries were localized in the outer medullary region in the present study.

Luft et al\textsuperscript{22} have shown that in transgenic rats producing excess renin (dTGR model), increased renal oxidative stress can increase NF-κB activity and induce adhesion molecules leading to renal interstitial fibrosis. Tubular ischemia has also been shown to induce the growth factor TGF-β, which has been shown to be induced by hypoxia in renal fibroblasts.\textsuperscript{27} The greater expression of TGF-β and NF-κB observed in the outer medulla of the kidneys exposed to the high levels of arterial pressure in the present studies would therefore be expected to lead to the increased expression of extracellular matrix proteins, such as collagen and fibronectin, and induce renal fibrosis.

The major portion of renal injury appeared to be pressure-dependent in this model. Only \(\approx 25\)\% of the interstitial fibrosis and tubular necrosis could be accounted for by the direct effects of Ang II. Some changes in the renal structure and function would be predicted by our recent study showing that even a nonhypertensive dose of Ang II (5 ng/kg minute intravenous) chronically administered for 7 days in rats resulted in increased expression of many genes related to pathways of oxidative stress, fibrosis, and apoptosis, as determined by cDNA microarrays and validated by Northern blot analysis.\textsuperscript{28} Furthermore, we have shown that Ang II can increase oxidative stress in medullary thick ascending limb and vasa recta in the freshly isolated nonperfused microtissue strips of outer medulla using real-time fluorescent superoxide detection,\textsuperscript{23} also indicating that Ang II can induce oxidative stress independent of changes in perfusion pressure. However, the Ang II-induced oxidative stress in the present model appears to have been greatly enhanced by increased RPP in the uncontrolled kidney, and this is the most important finding of the present study. It is assumed that the mild glomerular injury observed in the outer cortex of the uncontrolled kidney was the direct effect of circulating Ang II, whereas the more severe injuries of the juxtamedullary glomeruli were caused by the elevated pressure because this injury was significantly reduced in kidneys protected by servo-control pressure.

The present model system cannot separate the potential changes of local renal paracrine factors such as local Ang II production in the kidney. However, previous studies have shown that the non-clipped kidney of the 2-kidney 1-clip Goldblatt hypertensive rat model exhibits similar kidney tissue levels of Ang II compared with the clipped kidney,\textsuperscript{29} indicating that perhaps the intrarenal levels of Ang II of the uncontrolled and controlled kidneys of the present study were similar. Although the initial small increase in pressure on day 1 of the Ang II infusion (before turning on the servo-control device) may itself have had some effect, both kidneys were exposed to the same changes of pressure. The effect would have been the same to both kidneys until such time as the servo-control was turned on and pressure then normalized to control levels in the left kidney. This was the advantage of the paired comparison analysis of the present study. In addition, because we recognize that immunohistochemistry and imaging techniques are at best semi-quantitative in nature, and that the estimated proportion of pressure and Ang II-induced injury contains unpaired analysis using sham-operated rats, the present study used multiple methods and indicators for identifying renal damage.

The cortical glomerular capillary vessels are normally well protected from elevations of arterial pressure by autoregulatory mechanisms of the kidney,\textsuperscript{14,15} including myogenic responses of the afferent arteriole and tubulo-glomerular feedback.\textsuperscript{14} Upstream preglobular vessels such as the interlobular vessels, however, are exposed to elevations of RPP and are less well protected. The results of the present study indicate that the large portion of the injury observed in pregglomerular vessels was initiated by the pressure and not by Ang II.

It is also possible that juxtamedullary tubular injury caused by pressure-induced vasa recta endothelial injury could reduce tubulo-glomerular feedback and attenuate autoregulation. Attenuation of autoregulation in juxtamedullary glomeruli would further reduce autoregulation in the downstream medullary circulation and lead to a vicious cycle (tubulo-glomerular vicious cycle, Figure 6). We hypothesize that the tubular injury progresses to tubular stenosis, resulting in elevations of glomerular capillary pressure that would lead to glomerular sclerosis. This would explain why we have seen the severe glomerular sclerosis largely in the regions where pressure-related medullary tubular necrosis was seen in this study. It has been shown in norepinephrine-induced hypertension,\textsuperscript{30} Ang II-induced hypertension,\textsuperscript{5} and in the 2-kidney, 1-clip\textsuperscript{6} hypertension that renal injury occurs preferentially in the juxtamedullary region.\textsuperscript{18} Our results are consistent with the hypothesis proposed by Johnson et al\textsuperscript{18} that episodic hypertension can cause tubulo-interstitial injury and contribute to salt-sensitive hypertension. It has been previously demonstrated that medullary dysfunction can shift the pressure-natriuresis-diuresis relationship and lead to salt-sensitive hypertension.\textsuperscript{17} Taken together with the results of the present study, we conclude that the juxtamedullary nephrons of the renal outer medulla are the first targets of pressure-induced renal injury and lead to a further increase of arterial pressure and exacerbation of renal injury.

**Perspectives**

In this Ang II-induced model of hypertension, most of the renal injury was prevented when the kidney was protected from the increase of renal arterial perfusion pressure. Although some Ang II-dependent renal injury remained, the
largest portion of the juxtamedullary glomeruli, tubular necrosis, interstitial fibrosis, and interlobular artery injury were pressure-dependent. The nephrons of the juxtamedullary glomeruli were the first target of this injury. As we hypothesize in Figure 6, we believe that this injury may be explained by less protection because of reduced autoregulation capacity of medullary circulation. As medullary capillary dysfunction occurs by transmission of the pressure, the tubules in this region would be subjected to increased hypoxia. Tubular ischemia would induce oxidative stress in interstitial space and progress to fibrosis. The resulting oxidative stress and superoxide levels would reduce the bioavailability of NO and reduce the tubulovascular NO cross-talk mechanism that normally buffers the Ang II vasoconstriction of the vasa recta. All of these events we predict could lead to a vicious cycle producing tubular necrosis and interstitial fibrosis within the medulla, and juxtamedullary glomerular injury and acceleration of the severity of hypertension. The implications of these results regarding antihypertensive therapies are apparent.

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