Cortical Microvascular Remodeling in the Stenotic Kidney
Role of Increased Oxidative Stress

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Objective—Mechanisms of renal injury distal to renal artery stenosis (RAS) remain unclear. We tested the hypothesis that it involves microvascular remodeling consequent to increased oxidative stress.

Methods and Results—Three groups of pigs (n=6 each) were studied after 12 weeks of RAS, RAS+antioxidant supplementation (100 IU/kg vitamin E and 1 g vitamin C daily), or controls. The spatial density and tortuosity of renal microvessels (<500 μm) were tomographically determined by 3D microcomputed tomography. The in situ production of superoxide anion and the expression of vascular endothelial growth factor (VEGF), its receptor VEGFR-2, hypoxia-inducible-factor (HIF)-1α, von Hippel-Lindau (VHL) protein, and NAD(P)H oxidase (p47phox and p67phox subunits) were determined in cortical tissue. RAS and RAS+antioxidant groups had similar degrees of stenosis and hypertension. The RAS group showed a decrease in spatial density of cortical microvessels, which was normalized in the RAS+antioxidant group, as was arteriolar tortuosity. RAS kidneys also showed tissue fibrosis (by trichrome and Sirius red staining), increased superoxide anion abundance, NAD(P)H oxidase, VHL protein, and HIF-1α mRNA expression. In contrast, expression of HIF-1α, VEGF, and VEGFR-2 protein was downregulated. These were all significantly improved by antioxidant intervention.

Conclusions—Increased oxidative stress in the stenotic kidney alters growth factor activity and plays an important role in renal microvascular remodeling, which can be prevented by chronic antioxidant intervention. (Arterioscler Thromb Vasc Biol. 2004;24:1854-1859.)

Key Words: renal artery stenosis □ oxidative stress □ vascular biology □ free radicals/free-radical scavenger

Renal artery stenosis (RAS) is the major cause of renovascular hypertension and has become an important contributing cause to end-stage renal disease, especially in older patients. Chronic reduction of renal perfusion is often accompanied by renal atrophy, focal tubular necrosis, and interstitial fibrosis, but its impact on microvascular structure has not been fully characterized.

The construction and maintenance of the integrity of the microvascular network are usually driven by growth factors, like vascular endothelial growth factor (VEGF), and signaling through its type 2 receptor (VEGFR-2). VEGF is one of the best-studied, potent, angiogenic growth factors that stimulate endothelial cell proliferation and migration in vitro and angiogenesis in vivo, regulated mainly through hypoxia-inducible factor (HIF)-1α, whereas, in turn, von Hippel-Lindau (VHL) protein has a critical role in the regulation of HIF-1α. However, it is yet unknown whether microvascular remodeling in RAS is associated with modulation of VEGF expression.

In recent years, accumulating evidence has clearly indicated that increased oxidative stress mediates several aspects of irreversible renal injury and renal functional impairment. Increased intrarenal activity of angiotensin II in RAS is a potent stimulus for NAD(P)H oxidase, the main source of superoxide anion, a reactive oxygen species (ROS), which impairs endothelial function and participates in several reactions and signaling cascades leading to renal damage. Furthermore, kidneys exposed to chronic ischemia have diminished levels of endogenous free-radical scavengers like superoxide dismutase (SOD) and an impaired ability for cellular repair. We have previously shown that experimental RAS was associated with increased oxidative stress, intra-renal inflammation, and fibrosis. Increased oxidative stress may also modulate the expression of HIF-1 and VEGF and may therefore conceivably modulate changes in microvascular architecture as well.

Nevertheless, the microvascular architectural alterations in the stenotic kidney and the potential involvement of increased oxidative stress remain unclear, partly because of technical difficulties in studying 3D microvascular structure. Microcomputed tomography (micro-CT) is a powerful imaging

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technique that permits assessment of the 3D pattern of microvascular structure in situ and provides a unique and useful means for study of the spatial distribution and connectivity of microvessels within an organ. Indeed, we have demonstrated the feasibility of studying renal, myocardial, and vasa vasorum microvascular architecture with μ-CT in various pathophysiologic states.

Therefore, this study was designed to test the hypothesis that RAS leads to alterations in cortical microvascular structure that are mediated by increased oxidative-stress and would hence be attenuated by chronic antioxidant intervention.

Methods

This study was approved by the Institutional Animal Care and Use Committee. Three groups (n = 6 each) of age- and body weight-matched, female domestic pigs (55 to 65 kg) were studied after 12 weeks of no intervention (normal), RAS, or RAS with antioxidant supplementation (100 IU/kg vitamin E and 1 g vitamin C in the diet daily). We have previously shown that this combination was effective in decreasing oxidative stress and in normalizing vascular function.12,19 RAS was achieved by a local, irritant coil placed in the main renal artery at baseline, which induced gradual development of unilateral RAS, as previously described.20,21 The degree of RAS (reduction in lumen diameter) was subsequently measured by quantitative renal angiography.19 Mean arterial pressure measurement was obtained in all animals by use of a PhysioTel telemetry system (Data Sciences) implanted at baseline in the left femoral artery.20

Renal Hemodynamics and Function

Evaluation of regional renal function distal to the RAS was achieved in vivo by electron-beam CT.21 In vivo electron-beam CT flow studies were performed as previously detailed20,22 for assessment of renal blood flow (RBF) and glomerular filtration rate (GFR). A central venous catheter was inserted through the left jugular vein and served for contrast injections and sample collection.

Blood samples were collected from the inferior vena cava for measurement of serum creatinine (spectrophotometry) and systemic SOD activity (SOD assay kit, Cayman Inc), following vendor instructions, as we previously detailed.12 After euthanasia by pentobarbital sodium administration (100 mg/kg IV; Sleepaway, Ft Dodge Laboratories), the kidney was removed for in vitro studies.

Micro-CT Procedure

The stenotic kidney samples were prepared and scanned as previously described.14,15,17 Images were digitized for reconstruction of 3D volume images and analyzed with the Analyze software package (Biomedical Imaging Resource, Mayo Clinic, Rochester, Minn). For analysis of the cortex, the 3D tomographic images were oriented so that the z axis was parallel to the radial vessels. Based on the number of cortical sections, the cortex was tomographically divided into 10 levels obtained at equal intervals, starting at the juxtamedullary cortex. For analysis, levels 1 through 3 were considered as the inner third, levels 4 through 7 as the middle third, and levels 8 through 10 as the outer third of the cortex; for each third, the values from individual levels were averaged. The spatial density, average diameter, and vascular volume fraction (the ratio of the sum of cross-sectional areas of all vessels and the total area of the region of interest) of cortical microvessels (diameters <500 μm) were calculated in each level. Tortuosity (elongation factor) was estimated as we have recently described.22 In brief, 1 to 3 intracortical arterioles and their branches were tomographically “dissected” in each pig, the 3D path distance (total length) and linear distance (shortest distance between end points) of the main branches were calculated, and the elongation factor calculated by dividing path distance by linear distance.22

Renal Tissue

Expression of HIF-1α mRNA was determined by real-time quantitative reverse transcription–polymerase chain reaction (PCR), whereas protein expression of HIF-1α, VHL, VEGF, VEGFR-2, and p47phox and p67phox, subunits of NAD(P)H oxidase was detected by Western blotting. Superoxide anion was investigated in renal tissue by dihydroethidium (DHE) staining and fluorescence microscopy. To explore the mechanism of cell loss in RAS, apoptosis was evaluated by the terminal dUTP nick end-labeling (TUNEL) test.

Real-Time Quantitative PCR

Total RNA was isolated from kidneys with the TRIZOL (Invitrogen) method. cDNA was synthesized with the Invitrogen SuperScript first-strand synthesis kit, as we have recently described.22 To investigate the expression of HIF-1α mRNA, real-time PCR (DNA engine OPTICON, MJ Research) was subsequently performed with a SYBR Green JumpStart Taq ReadyMix kit (Sigma), as we have recently described.22 The porcine gene–specific sequence of HIF-1α primer was (left) 5′-TGC trichloroacetic acid (TCA) TCA GTT GCC ACT TC-3′ and (right) 5′-AAC AAT TAA TCT CCT GCC CT-3′. The relative amount of HIF-1α mRNA was normalized to an internal control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and relative to a calibrator (normal), calculated by

\[ \frac{2^{-\Delta\Delta C_T}}{ } \]

The sequence of the GAPDH primer was (upper) 5′-GGG CAT GAA CCA TGA GAA GT-3′ and (lower) 5′-GTC TCC TGG GTG GCA GTG AT-3′

Western Blotting

Equal protein (50 to 100 μg) of kidney homogenate was dissolved in sodium dodecyl sulfate–polyacrylamide gels (12% or 4% to 20%) under reducing conditions and electrophoretically transferred onto polyvinylidene difluoride membranes (Bio-Rad). Membranes were blocked for 1 hour in TBST/5% nonfat milk and incubated overnight at 4°C with antibodies against HIF-1α (1:1000, Novus Biologicals), VHL, p47phox, p67phox, VEGF (1:200, Santa Cruz Biotechnology), and VEGFR-2 (1:200, NeoMarkers). After being washed with TBST, the membranes were incubated for 1 hour with horseradish peroxidase–linked anti-rabbit antibody (1:5000, Amersham Pharmacia Biotech) in TBST/5% milk, and proteins were visualized by electrochemiluminescence. β-Actin (1:1000, Sigma) was used as the loading control.

DHE Staining

In situ production of superoxide anion was assessed in 30-μm frozen kidney sections with the oxidative fluorescent dye DHE, as we have previously described.23 Serial sections were equilibrated under identical conditions for 30 minutes at 37°C in Krebs’-HEPES buffer. Then, fresh buffer containing DHE (2 μmol/L) was applied onto each section, which was then coveredslipped, incubated for 30 minutes in a light-protected humidified chamber at 37°C, and evaluated by fluorescence microscopy.

Histology

Fixed blocks of tissue were embedded in paraffin, and 5-μm-thick sections were cut from each block. The sections were stained with trichrome and Sirius red. Corticomedullary cross sections of the ischemic kidney (1 per animal) were examined randomly by a computer-aided image analysis program (MetaMorph, Meta Imaging Series 4.6). In each representative slide, staining was semiautomatically quantified in 15 to 20 fields, expressed as percentage of staining of total surface area, and the results from all fields were averaged. Apoptotic signs were characterized in renal cells with the TUNEL method (ApopTag peroxidase in situ apoptosis detection kit, Serologicals Corp), as we have previously described.11 Apoptotic cells were quantified in 15 fields as the percentage of TUNEL-positive nuclei of total nuclei in each field.

Statistical Analysis

Continuous data are expressed as mean±SEM. Multiple group comparisons used ANOVA, followed by an unpaired t test, when
Cortical microvascular architecture and number

Cortical microvessels (<500 µm) that were clearly distinguishable by micro-CT and could be counted were as small as 80 µm in diameter. Several branching orders of radial vessels extended from the arcuate vessels peripherally, ending in numerous small vessels in the outer third of the cortex (Figure 1). Microvessels appeared less densely packed in stenotic RAS than in normal kidneys (Figure 1, top), and their spatial density was indeed significantly lower than normal, especially those microvessels <200 µm (Figure 2a) residing in the inner and outer cortex (Figure 2b). The vascular volume fraction and average diameter of cortical microvessels were also significantly decreased in RAS (Figure 2c and 2d). Antioxidant intervention normalized many of the changes in the RAS+antioxidant group (Table), particularly in vessels smaller than 100 µm (Figure 2a) and mainly in the outer cortex (Figure 2b). In both RAS and RAS+antioxidant groups, the number of vessels larger than 100 µm was significant lower than in the normal kidneys (Figure 2a), giving the impression that the vessels in these groups were narrower than in the normal group (Figure 1). However, the reduced diameter only reached significance in the untreated RAS group (Figure 2d). Moreover, tomographically isolated microvascular trees showed fewer branches that were more tortuous in RAS than in normal kidney (Table and Figure 1, bottom). These alterations were prevented by antioxidant intervention.

Redox Status

Compared with normals, RAS pigs showed decreased systemic activity of SOD, implying blunted radical-scavenging activity that was normalized in the RAS+antioxidant group (Table). Additionally, RAS increased renal p47phox and p67phox (the densitometry of p67phox was similar to p47phox) expression (Figure 3), suggesting increased potential for generation of superoxide, and was consistently accompanied by increased superoxide presence in renal tissue, as indicated by increased red fluorescence under DHE staining (Figure 4). All these changes were also attenuated by antioxidant intervention (Figures 3 and 4).

The RAS kidney showed increased expression of HIF-1α mRNA (Figure 3), which may reflect cortical response to tissue hypoxia. However, the protein expression of HIF-1α was decreased in RAS. This may be because of destabilization of HIF-1α mRNA by VHL or degradation of HIF-1α protein by superoxide, which were both elevated in the RAS kidney. VEGF and VEGFR-2 were all downregulated. Interestingly, although HIF-1α mRNA expression was similar in both RAS groups (Figure 3), protein expression of VHL, HIF-1α, VEGF, and VEGFR-2 was preserved in RAS animals that had been chronically supplemented with antioxidants.

In addition, the stenotic kidney showed more cellular apoptosis (mainly in vascular and tubular cells) compared with normal (Figure 4), as well as increased Sirius red (Figure I, available online at http://atvb.ahajournals.org) and trichrome staining (Figure 4), indicating increased fibrosis in the stenotic kidney. Focal ischemia, necrosis, and fibrosis were observed in RAS kidneys (Figure I). Both of these remodeling processes were significantly attenuated by antioxidant supplementation.
Discussion

This study demonstrated that alterations in renal microvascular architecture invoked by RAS are accompanied by increased oxidative stress and preserved by chronic antioxidant intervention, in association with improved RBF and renal fibrosis. These findings underscore a role for increased oxidative stress in modulating intrarenal microvascular architecture and function in the stenotic kidney.

RAS is an increasingly recognized cause of end-stage renal disease, but microvascular damage in RAS has not been characterized in detail. Limited histologic observation indicated glomerular and peritubular capillary injury in the ischemic kidney in some clinical cases of RAS and in a swine graded renal ischemic model. The current study describes alterations of microvascular architecture observed in RAS by using a powerful 3D imaging technique. Indeed, vascular rarefaction is known to occur in other ischemic organs, such as the hind limb or heart.

The degree of stenosis was not different between the 2 RAS groups, implicating intrarenal mechanisms in the observed microvascular remodeling in the stenotic kidney. A decrease in renal perfusion pressure or ischemia in the underperfused areas of the kidney may lead to cell swelling and occlusion of small vessels. Consequently, there may be loss of glomeruli and their nephrons, inflammation, and fibrosis of the ischemic areas. The increased tortuosity in RAS probably also represents vascular growth constrained by interstitial fibrosis, and this was also improved by intervention with antioxidants. Indeed, the decrease in renal perfusion pressure in the RAS kidney can increase production of angiotensin II, which in turn stimulates NAD(P)H oxidase to generate superoxide anion (as shown by DHE staining) that produces several vasoactive and fibrogenic factors. Moreover, the decreased SOD activity in our RAS pigs implies that scavenging activity is attenuated as well and suggests an overall increased abundance of superoxide anion and a pro-oxidant shift. Both increased formation of superoxide radical and peroxynitrite further impair SOD activity and thereby lead to a vicious circle of increased oxidative stress.

Notably, ROS may act as direct vasoconstrictors, inflammatory mediators, and fibrogenic factors. In the ischemic kidney, they likely mediate mainly fibrosis and scarring, which consequently restrict and interfere with vessel forma-
transcription factor HIF. 

Increased abundance of HIF-1α mRNA in the kidney, as shown in this study, may represent an adaptive response of renal cells to low Po2,54 but HIF-1α mRNA may be destabilized by VHL.6 Moreover, increased intracellular superoxide levels induce HIF-1α protein degradation by activating ubiquitin-proteasome,35 and NAD(P)H oxidase activity may importantly contribute to this posttranscriptional regulation of HIF-1α.36 Indeed, we have previously observed increased ubiquitin expression in RAS kidneys.11 In accordance with these observations, in the current study we found that decreased expression of VEGF was associated with downregulation of HIF-1α protein and upregulation of VHL, which may ultimately lead to microvascular loss in RAS. Therefore, antioxidant intervention may have also allowed neovascularization by preventing HIF-1α protein degradation.

In this study, RAS decreased microvascular density in both the outer and inner cortex. This may be because of a regional propensity and/or sensitivity to ischemia, as we have previously shown that these cortical regions have a low perfusion profile relative to the middle cortical zones.37 Antioxidant intervention succeeded in preserving microvascular density in the outer cortex, which is less sensitive to ischemia than the deep cortex.14

In summary, we observed that renal microvascular remodeling in the stenotic kidney was associated with increased renal tissue fibrosis, oxidative stress, apoptosis, and downregulation of HIF-1α and VEGF protein expression. Furthermore, the present study demonstrates that these changes are to a large extent redox-sensitive, because antioxidant supplementation not only decreased NAD(P)H oxidase expression and restored SOD activity and growth factor expression in RAS but also preserved microvascular density as well. Restoration of RBF by antioxidant supplementation implies that the ability of antioxidants to increase the number of vessels might have had beneficial effects on renal hemodynamics in vivo. The mechanisms underlying the increase of VHL expression in RAS need to be further investigated. Hence, this study supports an important role of increased

Figure 4. Top, Increased superoxide presence in renal tissue of RAS, as indicated by increased red fluorescence in DHE staining. Middle, TUNEL staining (×40) showing brown nuclei (arrow) as apoptotic cells. Bottom, Renal fibrosis evaluated by trichrome staining (×20) in normal, RAS, and RAS+antioxidant pigs. Abbreviations are as defined in text.

The current study shows that the deceased vessel density in the stenotic kidney is associated with downregulated protein expression of VEGF and VEGFR-2, supporting the important role of VEGF in maintenance of the integrity of the microvasculature. VEGF expression is regulated by oxygen tension, other growth factors, oncogenes, and especially by the transcription factor HIF.6,33 Increased abundance of HIF-1α protein degrada-

In contrast, in hypercholesterolemia, ROS may largely mediate inflammation, and in the absence of vascular obstruction, ischemia and fibrosis are minimal. Therefore, as we have previously observed,15 in the hypercholesterolemic pig model antioxidant supplementation attenuated neovascularization, likely achieved mainly by decreased inflammation rather than fibrosis. Lastly, unlike hypercholesterolemia, this study shows that in RAS, VEGF protein is downregulated, which might also blunt neovascularization. Interestingly, the decrease in the number of vessels in the stenotic kidneys was associated with a decline in GFR and RBF. Treatment with antioxidants normalized the number of small vessels and thereby RBF but did not restore nephrons and thus failed to normalize GFR. We also observed that although RBF was reduced in RAS kidneys, renal perfusion (flow per gram) was not decreased compared with normal and was in fact increased in the RAS+antioxidant group. Renal shrinkage in response to decreased renal perfusion pressure and RBF maintains relatively sufficient renal perfusion until this mechanism is overwhelmed by very severe stenoses.7 In RAS+antioxidant kidneys, renal volume was not restored, but perfusion may have increased because of decreased formation of the vasoconstrictor superoxide. Furthermore, renal perfusion was greater than normal, possibly because antioxidants may have a vasodilatory effect in vivo, as we have previously shown.32 Nevertheless, the decrease in vessel number in RAS kidneys and the increase in RAS+antioxidant animals is likely structural rather than functional, because our sample preparation methods allow for maximal relaxation in all groups before fixation. Furthermore, our study suggests that apoptosis may also contribute to loss of vascular cells.

The current study shows that the deceased vessel density in the stenotic kidney is associated with decreased vessel density in the deep cortex.14 This increased red fluorescence in DHE staining. Middle, TUNEL staining (×40) showing brown nuclei (arrow) as apoptotic cells. Bottom, Renal fibrosis evaluated by trichrome staining (×20) in normal, RAS, and RAS+antioxidant pigs. Abbreviations are as defined in text.
oxidative stress in the pathogenesis of several facets of renal injury in RAS.

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