Mechanisms of Renal Structural Alterations in Combined Hypercholesterolemia and Renal Artery Stenosis


Objective—Atherosclerotic renovascular disease (ARVD) aggravates renal scarring more than other causes of renal artery stenosis (RAS), but the underlying pathogenic mechanisms of this potential profibrotic effect remain unclear. We tested the hypothesis that coexistence of atherosclerosis and RAS interferes with renal tissue remodeling.

Methods and Results—Single-kidney hemodynamics and function were quantified in vivo with electron-beam computed tomography in 3 groups of pigs (n=7 each): normal pigs, pigs 12 weeks after induction of unilateral RAS (RAS group), and pigs with similar-degree RAS fed a 12-week 2% hypercholesterolemic diet (HC+RAS, simulating early ARVD). Kidneys were studied ex vivo by Western blotting and immunohistochemistry. Renal volume, renal blood flow, and glomerular filtration rate were similarly decreased in RAS and HC+RAS ischemic kidneys, accompanied by similar increased expression of profibrotic factors like transforming growth factor-β, tissue inhibitor of metalloproteinase-1, and plasminogen activator inhibitor-1. Nevertheless, HC+RAS kidneys showed increased intrarenal fibrosis compared with RAS-only kidneys. Furthermore, expression of nuclear factor-κB was increased, expression of extracellular (matrix metalloproteinase-2) and intracellular (ubiquitin) protein degradation systems was decreased, and apoptosis was blunted.

Conclusions—Diet-induced HC superimposed on RAS accelerates the development of fibrosis in the stenotic kidney by amplifying profibrotic mechanisms and disrupting tissue remodeling. These alterations might contribute to renal disease progression in ARVD and might account for the increased propensity for end-stage renal disease. (Arterioscler Thromb Vasc Biol. 2003;23:1001-1008.)

Key Words: atherosclerosis ■ kidney ■ renal disease ■ fibrosis

During the past two decades, atherosclerosis has become the clinical entity responsible for as much as 90% of all cases of renovascular disease. Clinical studies suggest that 10% to 40% of elderly hypertensive patients with newly documented end-stage renal disease (ESRD) and no demonstrable, primary renal disease have significant atherosclerotic renovascular disease (ARVD), whose incidence is on the rise in the elderly population. In addition to atherosclerotic plaques in the main renal artery, atherosclerosis might also directly compromise the renal parenchyma and intrarenal vessels. Therefore, ARVD might aggravate renal hypoperfusion and tissue injury and compromise renal function more often than other causes of main-vessel renal artery stenosis (RAS).

Furthermore, renal revascularization alone restores kidney function only in a minority of cases of ARVD. The poor response to revascularization observed in ARVD has led to the speculation that synergism between atherogenic factors and hypoperfusion distal to the stenosis might accelerate progressive renal disease, fibrosis, and eventual ESRD. Renal scarring and tissue remodeling are dynamic processes that involve both synthesis and degradation of extracellular matrix (ECM), the balance between which might be disrupted in several renal diseases. Hence, matrix accumulation and fibrosis in RAS might result from either increased deposition or decreased degradation of the ECM. Interestingly, several proinflammatory and progrowth factors that have been implicated in renal tissue remodeling in RAS might also be activated in hypercholesterolemia (HC), a surrogate of early atherosclerosis and an independent risk factor for renal disease progression. In both conditions, renal functional impairment is accompanied by activation of the renin-angiotensin system and increased generation of reactive oxygen species, which can stimulate production and activity of various vascular, glomerular, and tubulointerstitial growth factors and redox-sensitive transcription factors that can modulate renal tissue injury. In addition, HC might further contribute to the progression of renal damage through deposition of atherogenic and oxidized lipoproteins and by...
interfering with matrix turnover and might therefore potentially promote ischemia and fibrosis in the stenotic kidney. We have recently shown in an animal model of concurrent HC and RAS, a surrogate of early chronic ARVD, that tubular and glomerular dysfunction were accentuated compared with HC or RAS alone. These functional alterations were accompanied by a marked increase in renal oxidative stress, inflammation, and tubular atrophy, as well as perivascular and particularly tubulointerstitial fibrosis in the stenotic HC+RAS kidneys compared with RAS-only kidneys. However, the mechanisms by which concurrent HC might accelerate irreversible renal tissue injury in HC+RAS have not been elucidated. Hence, the present study was designed to test the hypothesis that superimposed early atherosclerosis would amplify profibrotic events and mechanisms of tissue remodeling that might favor renal scarring.

**Methods**

**Animal Procedures**

All procedures were approved by the Institutional Animal Care and Use Committee. Twenty-one domestic pigs (55 to 65 kg) were studied after a 12-week treatment. Seven pigs (normal) were fed a normal diet, whereas in 7 others (RAS), a local-irritant coil placed in the left renal artery at baseline induced gradual development of unilateral RAS, as previously described, followed by a 12-week normal diet. In 7 others (HC+RAS), RAS was accompanied by a 12-week atherogenic diet of 2% cholesterol and 15% lard (TD-93296, Harlan-Teklad), as previously described. Blood pressure measurement was obtained in all animals by use of a telemetry system (PhysioTel, Data Sciences) implanted at baseline in the left carotid artery. Mean arterial pressure was recorded at 5-minute intervals and averaged for each 24-hour period. Levels reported were those obtained for 2 days before each in vivo study.

On the day of in vivo studies, each animal was anesthetized with 0.5 g ketamine and xylazine IM, intubated, and mechanically ventilated with room air. Anesthesia was maintained with a mixture of 0.5 g ketamine and xylazine (0.03 mg · kg⁻¹ · h⁻¹) and 15% lard (TD-93296, Harlan-Teklad), as previously described. Blood pressure measurement was obtained in all animals by use of a telemetry system (PhysioTel, Data Sciences) implanted at baseline in the left carotid artery. Mean arterial pressure was recorded at 5-minute intervals and averaged for each 24-hour period. Levels reported were those obtained for 2 days before each in vivo study.

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**Protein Extraction and Western Blotting**

Frozen renal tissue (including both cortex and medulla) was pulverized and homogenized at 4°C in chilled protein extraction buffer. The homogenate was incubated in buffer for 1 hour at 4°C, and the homogenized lysates were then centrifuged for 15 minutes at 14,000 rpm. The supernatant was removed and the protein concentration was determined by spectrophotometry with a protein assay (Coomassie Plus, Pierce). The lysate was then diluted 1:4 in 1× polyacrylamide gel electrophoresis sample buffer, sonicated, and heated at 95°C to denature the proteins. The lysate was then loaded onto a gel and subsequently run as per standard Western blotting protocols with the use specific antibodies against NF-kB (1:200, Santa Cruz Biotechnology Inc), MMP-2 (1:400, Chemicon International), TIMP-1 (1:200, Santa Cruz Biotechnology), and PAI-1 (1:200, Santa Cruz Biotechnology). The membrane was exposed for 5 minutes to a chemiluminescence developing system (for polyclonal antibodies, SuperSignal West Pico Chemiluminescent Substrate, Pierce; for monoclonal antibodies, ECL Western Blotting Detection reagents, Amersham Biosciences), and then finally exposed to x-ray film (Kodak), which was subsequently developed, and intensities of the protein bands were determined by densitometry.

**Immunohistochemistry**

Immunohistochemistry for TGF-β and LOX-1 was performed on frozen renal tissue with the monoclonal primary antibodies against TGF-β (1:100; MAB1835, R&D Systems) and LOX-1 (1:280), followed by use of a commercially available kit (Vectastain-Elite ABC kit, Vector Laboratories) according to the vendor’s instructions.

Immunohistochemistry for NF-kB, MMP-2, ubiquitin, TIMP-1, and PAI-1 was performed on deparaffinized renal tissue. Primary antibodies used were either polyclonal against TIMP-1 and PAI-1 (1:100 each, Santa Cruz Biotechnology) or monoclonal against NF-kB (1:50, Santa Cruz Biotechnology), MMP-2 (1:100, Chemicon International), and ubiquitin (1:200, Berkeley Antibody Co). The secondary antibody (IgG Envision Plus DAKO) was followed by staining with the use of a commercially available kit (Vector NovaRED substrate kit, Vector Laboratories), and the slides were counterstained with hematoxylin.

**TUNEL Assay**

As an index of apoptosis, the TUNEL method was performed on paraffin-embedded kidney tissue (Apoptag peroxidase in situ apoptosis detection kit, Intergen Co) according to the vendor’s instructions.

**Data Analysis**

For EBCT, manually traced regions of interest were selected from EBCT images of the aorta, renal cortex, and medulla, and their densities were sampled. Time-density curves, which show the contrast medium transit through the kidney, were generated and fitted with extended gamma-variable curve fits, and the area enclosed under each segment of the curve and its first moment were calculated by using curve-fitting parameters, as we have previously shown. These were used to calculate single-kidney GFR and RBF by using previously validated methods.

For histology, midhilar cross sections of the ischemic kidney (1 per animal) were examined with a computer-aided image analysis program (MetaMorph, Meta Imaging Series 4.6). In each representative slide, immunostaining in the renal cortex was semiautomatically quantified in 15 to 20 fields by the computer program and
expressed as a percentage of staining of total surface area; the results from all fields were then averaged. 13,17 For TUNEL, the fraction of apoptotic cells of the total number of cells was calculated in randomly selected fields from each kidney.

**Statistical Analysis**

Results are mean±SEM. Comparisons within groups were performed with a paired Student’s t test, and comparisons among groups were analyzed by ANOVA, with the Bonferroni correction for multiple comparisons, followed by an unpaired Student’s t test. Statistical significance was accepted for P≤0.05.

**Results**

Mean arterial pressure and renal vascular resistance in RAS and HC+RAS groups were similarly increased compared with normal animals, and serum cholesterol levels were elevated in HC+RAS pigs (Table 1). Circulating LDL showed increased oxidizability (shortened lag time and increased LDL malondialdehyde and LDL relative electrophoretic mobility) in HC+RAS compared with normal and RAS animals; Table 1 and Figure 1). Plasma creatinine was significantly elevated in RAS and HC+RAS stenotic kidneys compared with normal pigs, whereas plasma renin activity was not different among the groups. Renal volume and the degree of stenosis were not different among the ischemic kidneys (Table 1). RBF and GFR were similarly decreased in both RAS and HC+RAS stenotic kidneys compared with normal kidneys (Table 1), although renal perfusion (blood flow per

**TABLE 1. Systemic Characteristics, Single-Kidney Hemodynamics, and LDL-Oxidizability in Normal, Renal Artery Stenosis (RAS) and Hypercholesterolemic (HC)+RAS Pigs**

<table>
<thead>
<tr>
<th></th>
<th>Normal, n=7</th>
<th>RAS, n=7</th>
<th>HC+RAS, n=7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean arterial pressure, mm Hg</td>
<td>99.9±4.7</td>
<td>120.7±11.0*</td>
<td>123.7±6.7*</td>
</tr>
<tr>
<td>Renal vascular resistance</td>
<td>0.2±0.01</td>
<td>0.37±0.1*</td>
<td>0.4±0.05*</td>
</tr>
<tr>
<td>Total Cholesterol, mmol/L</td>
<td>1.83±0.1</td>
<td>1.83±0.2</td>
<td>8.76±0.4†</td>
</tr>
<tr>
<td>LDL, mmol/L</td>
<td>0.85±0.1</td>
<td>1.03±0.1</td>
<td>7.13±0.6†</td>
</tr>
<tr>
<td>LDL-Lag time, min</td>
<td>86±2.3</td>
<td>83.3±1.4</td>
<td>70.0±1.9†</td>
</tr>
<tr>
<td>LDL-MDA, nM/mg</td>
<td>6.3±0.4</td>
<td>7.0±0.2</td>
<td>9.3±0.2†</td>
</tr>
<tr>
<td>LDL-REM, mm</td>
<td>10.7±0.2</td>
<td>10.4±0.3</td>
<td>12.9±0.3*†</td>
</tr>
<tr>
<td>Creatinine, μmol/L</td>
<td>127.9±6.0</td>
<td>163±11.1*</td>
<td>181.1±20.9*</td>
</tr>
<tr>
<td>mg/dL</td>
<td>1.44±0.06</td>
<td>1.84±0.12*</td>
<td>2.04±0.24*</td>
</tr>
<tr>
<td>Plasma renin activity, ng/mL/h</td>
<td>0.37±0.1</td>
<td>0.31±0.07</td>
<td>0.36±0.1</td>
</tr>
<tr>
<td>Degree of stenosis, %</td>
<td>...</td>
<td>77.8±6.9*</td>
<td>71.0±10.6*</td>
</tr>
<tr>
<td>Kidney volume, mL</td>
<td>150.1±7.2</td>
<td>89.2±21.2*</td>
<td>101.0±7.1*</td>
</tr>
<tr>
<td>Renal blood flow, mL/min</td>
<td>553.4±48.7</td>
<td>326.5±87.4*</td>
<td>349.4±49.6*</td>
</tr>
<tr>
<td>Renal perfusion, mL/min/mL</td>
<td>3.68±0.3</td>
<td>3.38±0.3</td>
<td>3.43±0.3</td>
</tr>
<tr>
<td>Glomerular filtration rate, mL/min</td>
<td>69.1±3.3</td>
<td>47.9±10.2*</td>
<td>50.0±6.0*</td>
</tr>
</tbody>
</table>

Values are mean±SEM.

MDA indicates malondialdehyde; REM, relative electrophoretic mobility.

*P<0.05 vs normal. †P<0.05 vs RAS.

Figure 1. Representative renal staining for trichrome (a), ubiquitin (b), and TUNEL (c) in the ischemic kidneys of normal, RAS, and HC+RAS pigs (n=7 each). Superimposition of HC on RAS resulted in increased tubulointerstitial and glomerular fibrosis, accompanied by blunted renal defense mechanisms responsible for removal of excessive intracellular damaged proteins (ubiquitin) and cells (apoptosis) (Table 2). This pattern suggests a shift favoring renal scarring in HC+RAS. Magnification ×40.
cubic centimeter of tissue) was not different among the three groups, likely owing to compensatory renal shrinkage that tends to sustain renal perfusion.\textsuperscript{14}

Renal Tissue

Trichrome staining showed a significant increase in renal fibrosis in HC\textsubscript{1}RAS pigs compared with the other groups (Table 2 and Figure 1a), and was localized mainly in the peritubular interstitial space. Immunoblots and immunohistochemistry demonstrated that tubular (especially proximal tubule), interstitial, and glomerular protein expression of NF-κB (a stimulator of profibrotic cytokines) was markedly increased in HC+RAS pigs compared with the two other groups (Figure 2a; \( P<0.05 \)), indicating that superimposition of HC on RAS amplified NF-κB expression. Profibrotic TIMP-1 and PAI-1 were similarly and significantly increased in both RAS and HC+RAS compared with normal pigs, and immunohistochemistry showed that they were both localized in both the tubular and interstitial compartments (Figure 3a and 3b; \( P<0.05 \) for all). Finally, MMP-2 showed a significant increase compared with normal in both RAS and HC+RAS (\( P<0.05 \)) groups but showed a strong tendency to be lower compared with RAS (Figure 2b; \( P=0.061 \)), suggesting that superimposition of HC on RAS blunted the activation of this extracellular protein degradation system.

<table>
<thead>
<tr>
<th></th>
<th>Normal, ( n=7 )</th>
<th>RAS, ( n=7 )</th>
<th>HC+RAS, ( n=7 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trichrome</td>
<td>2.9±0.3</td>
<td>12.5±0.6*</td>
<td>16.4±0.4*†</td>
</tr>
<tr>
<td>TGF-β</td>
<td>5.2±0.4</td>
<td>9.3±0.2*</td>
<td>9.4±0.2*</td>
</tr>
<tr>
<td>LOX-1</td>
<td>2.3±0.1</td>
<td>3.7±0.3*</td>
<td>3.8±0.3*</td>
</tr>
<tr>
<td>Ubiquitin</td>
<td>3.2±0.3</td>
<td>5.7±0.1*</td>
<td>5.0±0.3*†</td>
</tr>
<tr>
<td>TUNEL</td>
<td>1.9±0.7</td>
<td>8.7±2.0*</td>
<td>1.8±0.2*</td>
</tr>
</tbody>
</table>

\( \text{TGF-β indicates transforming growth factor-β; LOX-1, lectin-like receptor type 1 for oxidized LDL; TUNEL, terminal dUTP nickend-labeling.} \)

\( *P<0.05 \) vs normal. \( †P<0.05 \) vs RAS.

Figure 2. Representative renal immunoblots and immunohistochemistry, demonstrating protein expression of NF-κB (a) and MMP-2 (b) in normal, RAS, and HC+RAS pigs (\( n=7 \) each). These show significantly increased tubular and glomerular activity of NF-κB in HC+RAS compared with both normal and RAS animals. However, the increased expression of MMP-2 induced by RAS alone was relatively blunted in HC+RAS, suggesting that the coexistence of HC+RAS led to attenuation in extracellular protein degradation and removal in the stenotic kidney. Magnification \( \times 40 \). \( *P<0.05 \) vs normal, \( †P<0.05 \) vs RAS, \( #P=0.061 \) vs RAS.
Tubular and glomerular expression of TGF-β was similarly increased in both the RAS and HC+RAS animals (Table 2 and Figure 4a), as was expression of LOX-1 in endothelial cells of intrarenal arterioles, suggesting increased potential for ox-LDL uptake (Table 2 and Figure 4b). On the other hand, the expression of ubiquitin (an intracellular protein degradation system) and the degree of tubular and glomerular (albeit not vascular) apoptosis were markedly increased in RAS compared with normal pigs but were significantly blunted in HC+RAS compared with RAS animals (Table 2 and Figure 1b and 1c), suggesting an overall imbalance in protein degradation and removal favoring tissue growth in HC+RAS kidneys.

Figure 3. Representative renal immunoblots and immunohistochemistry, demonstrating protein expression of TIMP-1 (a) and PAI-1 (b) in normal, RAS, and HC+RAS pigs (n=7 each), showing a similar increase in both RAS and HC+RAS compared with normal, suggesting an increased profibrotic activity. Magnification ×40. *P<0.05 vs normal.

Figure 4. Representative immunostaining for LOX-1 (a) and TGF-β (b) in kidneys of normal, RAS, and HC+RAS pigs (n=7 each). RAS and HC+RAS animals show a similar increase in LOX-1 expression in renal vascular endothelial cells and in tubular and glomerular expression of TGF-β. Magnification ×20.
Discussion

This study demonstrates that the combination of HC and RAS amplifies activation of mechanisms that can promote renal vascular, glomerular, and tubulointerstitial injury compared with RAS alone, despite similar renal hemodynamics. Both RAS and HC+RAS were associated with a marked increase in tubular and glomerular expression of profibrotic TGF-β, TIMP-1, and PAI-1. However, these were accompanied in HC+RAS by increased expression of NF-κB, an attenuated increase in the expression of MMP-2 and ubiquitin, and decreased apoptosis compared with RAS. This suggests a shift in the tissue remodeling process in HC+RAS that favors renal fibrosis and matrix accumulation. These alterations might play a role in the progression of atherosclerotic RAS and its propensity for ESRD.

ARVD has been increasingly recognized as a primary cause for ESRD, which is continuously increasing in prevalence. ARVD might cause renal failure and hypertension and is accompanied by high cardiovascular morbidity and mortality. Furthermore, clinical studies suggest that renal parenchymal damage is a major determinant of renal dysfunction in ARVD, and they demonstrate greater renal compromise and worse outcomes in this disease compared with RAS alone. However, the mechanisms by which tissue injury might become irreversible are not yet completely understood.

We have previously shown in a model of early, chronic ARVD the distinct detrimental effects on renal function and structure in vivo and in vitro. The current study extended our previous observations and further investigated the expression of growth-promoting factors likely to amplify renal injury and whose induction might be accelerated by concurrent early atherosclerosis and RAS. We observed that immunoreactivity of the profibrotic TGF-β, which is often involved in renal disease progression, was increased in both RAS and HC+RAS groups to a similar extent. Furthermore, we observed a markedly increased glomerular and tubulointerstitial expression of PAI-1 and TIMP-1 in both RAS and HC+RAS compared with normal animals. All of these factors can be induced by angiotensin II, reactive oxygen species, cytokines, and growth factors; play a pivotal role in growth-promoting activity, regulation of apoptosis, and induction of changes in cell morphology; and can promote glomerulosclerosis and tubulointerstitial fibrosis in renal disease. However, the similar increased expression of these profibrotic factors in the ischemic kidneys strongly suggests that additional factors might be involved in the amplified fibrosis observed in HC+RAS compared with RAS animals.

The increased expression of NF-κB in HC+RAS might conceivably contribute to this process. This transcription factor activates a variety of genes involved in inflammation and cell adhesion, proliferation, and survival, and its activity might be augmented by increased oxidative stress. Hence, the increase in renal inflammation in HC+RAS might promote fibrosis and scarring by increasing the production of fibrogenic factors that stimulate the synthesis of ECM proteins. In addition, superimposed HC might accelerate renal injury in HC+RAS through cytotoxic ox-LDL. We observed a similar immunoreactivity for LOX-1 that was accompanied by augmented oxidizability of LDL in HC+RAS compared with RAS pigs. A concomitant increase in the availability of ox-LDL and its receptor expression might thus facilitate ox-LDL uptake in HC+RAS more than in RAS alone. Ox-LDL might induce structural modifications of cell proteins, which subsequently might impair cell viability and lead to the generation and deposition of oxidized proteins.

Accumulation of oxidatively modified proteins can elicit cellular damage and is curtailed, under normal conditions, by intracellular protein degradation systems like the ubiquitin/proteasome system. Indeed, the current study shows that the expression of ubiquitin was increased in RAS but was relatively attenuated in HC+RAS. The latter might possibly be due to inhibition of ubiquitin by increased ox-LDL. Furthermore, decreased ubiquitin expression might affect activation of NF-κB, because degradation of its inhibitor, IκB, by the proteasome system is one of the main pathways for NF-κB activation. Hence, the increased expression of NF-κB in HC+RAS might be a compensatory mechanism or might reflect NF-κB activation by alternative pathways.

In addition, renal growth and accumulation of ECM is a part of the dynamic process observed in renal development and disease and is normally counterbalanced by ECM degradation. The major regulators of ECM degradation in the kidney are the MMPs. Indeed, the expression of MMP-2, which is synthesized by both glomerular epithelial and mesangial cells, was increased in the ischemic RAS and HC+RAS kidneys, likely as a compensatory mechanism to offset ECM accumulation and renal scarring. Nevertheless, renal expression of MMP-2 strongly tended to be lower in HC+RAS kidneys compared with RAS. These observations imply that superimposed HC not only promotes renal fibrosis and ECM deposition but might also attenuate extracellular and intracellular protein degradation and removal in HC+RAS.

A prominent adaptive response to minimize necrosis in the face of ensuing cellular damage is apoptosis, a programmed cell death designed to eliminate superfluous or harmful cells, which is regulated by several proapoptotic and antiapoptotic factors. Indeed, the ischemic RAS kidneys showed increased tubular and glomerular apoptosis, which might serve as a protective mechanism. However, in the milieu of concurrent HC+RAS, this defensive mechanism was attenuated, perhaps consequent to additive antiapoptotic effects attributed to the increased activation of NF-κB and decreased ubiquitin. Furthermore, it has been recently shown that ox-LDL can promote cell lysis in a pathway independent of apoptosis and is more likely to induce tissue injury. This observation supports the notion that concurrent HC might not only activate injurious mechanisms but might also, in parallel, blunt a hierarchy of defense mechanisms meant to limit injury in the ischemic kidney, resulting in a shift to favor the scarring process. On the other hand, HC might increase coronary arterial apoptosis, but this was not observed in intrarenal microvessels. Speculatively, apoptosis might precede or parallel atherosclerotic structural changes that are often absent in the microcirculation. The precise effects of atherosclerosis on cell cycle events, apoptosis, or...
the mechanisms responsible for attenuating MMP expression in the ischemic HC+RAS kidney need further studies.

In summary, the present study demonstrates that interaction between early atherosclerosis and hyperperfusion interferes with renal tissue remodeling, resulting in the augmented intrarenal fibrosis observed in the HC+RAS stenotic kidney. Although at this early stage renal hemodynamics and function declined to the same degree in RAS and HC+RAS, the increased tissue injury is likely to accelerate deterioration of renal function at a more advanced phase. Identifying and modifying the pathways of renal fibrosis in the setting of vascular compromise will be essential to plan rational management of this disorder. These studies indicate the potential for modulation by tools aimed at limiting oxidative stress, lipid peroxidation, and those pathways regulating apoptosis. These alterations are likely to play a pivotal role in promoting renal disease progression in the context of atherosclerotic renovascular disease and might account for the increase propensity for ESRD.

Acknowledgments

This study was supported by grant number HL-63282 from the National Institutes of Health, the American Heart Association, and ISNIH 99.56980 (to C.N.).

References

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Arterioscler Thromb Vasc Biol. published online May 15, 2003;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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