Distinct Renal Injury in Early Atherosclerosis and Renovascular Disease

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Background—Atherosclerotic renovascular disease may augment deterioration of renal function and ischemic nephropathy compared with other causes of renal artery stenosis (RAS), but the underlying mechanisms remain unclear. This study was designed to test the hypothesis that concurrent early atherosclerosis and hypoperfusion might have greater early deleterious effects on the function and structure of the stenotic kidney.

Methods and Results—Regional renal hemodynamics and function at baseline and during vasoactive challenge (acetylcholine or sodium nitroprusside) were quantified in vivo in pigs by electron-beam computed tomography after a 12-week normal (n=7) or hypercholesterolemic (HC, n=7) diet. RAS (n=6), or concurrent HC and a similar degree of RAS (HC+RAS, n=7). Flash-frozen renal tissue was studied ex vivo. Basal cortical perfusion and single-kidney glomerular filtration rate (GFR) were decreased similarly in the stenotic RAS and HC+RAS kidneys, but tubular fluid reabsorption was markedly impaired only in HC+RAS. Perfusion responses to challenge were similarly blunted in the experimental groups. Stimulated GFR increased in normal, HC, and RAS (38.3±3.6%, 36.4±7.6%, and 60.4±9.3%, respectively, P<0.05), but not in HC+RAS (6.5±15.1%). These functional abnormalities in HC+RAS were accompanied by augmented perivascular, tubulointerstitial, and glomerular fibrosclerosis, inflammation, systemic and tissue oxidative stress, and tubular expression of nuclear factor-κB and inducible nitric oxide synthase.

Conclusions—Early chronic HC+RAS imposes distinct detrimental effects on renal function and structure in vivo and in vitro, evident primarily in the tubular and glomerular compartments. Increased oxidative stress may be involved in the proinflammatory and progrowth changes observed in the stenotic HC+RAS kidney, which might potentially facilitate the clinically observed progression to end-stage renal disease. (Circulation. 2002;106:1165-1171.)

Key Words: atherosclerosis ▪ kidney ▪ regional blood flow

Renal artery stenosis (RAS) is the most common cause for secondary hypertension and may lead to deterioration of renal function and renal tissue injury (“ischemic nephropa-thy”).1 Up to 90% of stenotic lesions are caused by athero-sclerosis,2 the prevalence of which as an underlying cause for end-stage renal disease (ESRD) is on the rise, especially in the elderly population.1,3 Hence, there is a pressing need to define the underlying mechanisms responsible for renal damage in this disease. Notably, compared with fibromuscu-lar dysplasia, atherosclerotic RAS is associated with poorer outcomes in terms of hypertension and renal function,4,5 suggesting that atherogenic factors other than stenosis itself are involved in the mechanism of renal injury in atheroscler-otic RAS.2,6 However, these factors are yet to be identified.

Renal injury in RAS may be initiated by a reduction in nitric oxide (NO) bioavailability and increased intrarenal activity of the renin-angiotensin system, resulting in inflamma-
ocinity and the predominance of vasoconstrictors and growth-promoting factors.7–9 Similar alterations also characterize atherosclerosis and hypercholesterolemia (HC), which are risk factors for progression of renal disease.10,11 Furthermore, both ischemic nephropathy and HC are associated with increased generation of reactive oxygen species (ROSs) and other radicals or increased oxidative stress. ROSs may further interact with NO and decrease its bioavailability, thereby generating the pro-oxidant peroxynitrite, and may impair intrarenal vascular, glomerular, and tubular function and increase the activity of growth factors and inflammatory mediators, eventuating in renal structural damage. How-
ever, whether these detrimental effects are exacerbated in atherosclerotic RAS by a cross talk between HC and hypoperfusion remains to be defined. In particular, their interaction to affect intrarenal hemodynamics and function in vivo has been difficult to assess because of lack of...
reliable, noninvasive techniques capable of studying the kidney distal to RAS.

Electron-beam computed tomography (EBCT) provides accurate, reproducible, simultaneous, and noninvasive quantifications of single-kidney volume, perfusion, glomerular filtration rate (GFR), and segmental tubular function,12 which are difficult to obtain using other methods.13 Hence, the present study was designed to investigate the effects of HC+RAS on the stenotic kidney in vivo and to test whether early chronic HC+/H11001 RAS induces more pronounced increases in oxidative stress and renal hemodynamic, functional, and structural impairment than hypoperfusion or HC alone.

Methods
All procedures were approved by the Institutional Animal Care and Use Committee. Twenty-seven domestic pigs (57 to 67 kg; Pork Partners, Stewartville, Minn) were studied after a 12-week treatment. Seven pigs (normal) were fed a normal diet, and 7 others (HC) received an atherogenic diet of 2% cholesterol and 15% lard (TD-93296, Harlan-Teklad). In 6 others (RAS), a local-irritant coil placed in the left renal artery at baseline induced gradual development of unilateral RAS, as previously described,15 followed by a 12-week normal diet. In 7 others (HC+RAS), a 12-week HC diet and RAS were initiated simultaneously.

The degree of RAS was subsequently measured by quantitative renal angiography,13 and blood samples were collected from the inferior vena cava and renal veins bilaterally for measurement of plasma renin activity (PRA). In vivo EBCT flow studies were then performed for assessment of basal regional renal perfusion, renal blood flow (RBF), GFR, and tubular function and were repeated during supraprenal infusion of acetylaceholine (ACh) and sodium nitroprusside (SNP) to test endothelium-dependent and -independent responses, respectively.

In vitro studies were subsequently performed to obtain plasma lipid profiles (Roche), PRA (New England Nuclear), and serum creatinine (spectrophotometry). Plasma oxidation was assessed spectrophotometrically by the lag time, malondialdehyde content, and creatinine (spectrophotometry). Plasma oxidation was assessed spectrophotometrically by the lag time, malondialdehyde content, and creatinine (spectrophotometry).

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In normal animals, ACh significantly increased RBF and GFR (to 69.2 ± 5.1 mL/min) but not RBF or perfusion. In RAS, cortical perfusion increased (to 42.7 ± 4.2 mL/min) but not medullary perfusion. In HC, ACh induced a similar and significant increase in RBF, GFR, or any regional perfusion. Furthermore, the GFR response tended to be further blunted compared with HC or RAS (P = 0.08 and 0.07, respectively).

In normal and RAS kidneys, ITC decreased significantly in the distal (P < 0.05, data not shown) but remained unchanged in the proximal nephron. In HC, ACh induced a similar and significant decrease in ITC in the distal (P = 0.02 but in the proximal nephron as well (P = 0.02). In contrast, in HC + RAS, ITC remained unchanged along the nephron.

### Response to ACh and SNP

In normal animals, ACh significantly increased RBF and GFR (to 735.2 ± 53.9 and 97.5 ± 9.3 mL/min, respectively, Figure 1a, b and c) and cortical, medullary, and papillary perfusion (to 5.69 ± 0.54, 4.22 ± 0.38, and 3.87 ± 0.67 mL·min⁻¹·g⁻¹, respectively, P ≥ 0.02 for each). In HC, ACh significantly increased GFR (to 69.2 ± 5.1 mL/min) but not RBF or perfusion. In RAS, cortical perfusion increased (to 42.7 ± 4.2 mL·min⁻¹·g⁻¹, P = 0.04), as did GFR (to 42.7 ± 17 mL/min, Figure 1c), but RBF and medullary perfusion remained unchanged. In HC + RAS, however, ACh failed to increase RBF, GFR, or any regional perfusion. Furthermore, the GFR response tended to be further blunted compared with HC or RAS (P = 0.08 and 0.07, respectively).

In normal and RAS kidneys, ITC decreased significantly in the distal (P < 0.05, data not shown) but remained unchanged in the proximal nephron. In HC, ACh induced a similar and significant decrease in ITC in the distal (P = 0.02) but in the proximal nephron as well (P = 0.02). In contrast, in HC + RAS, ITC remained unchanged along the nephron.

| Table 1. Systemic Characteristics, Redox Status, and Tissue Activities of Radical Scavengers in Normal, HC, RAS, and HC + RAS Pigs |
|-----------------|-----------------|-----------------|-----------------|
|                 | Normal          | HC              | RAS             | HC + RAS        |
|                 | (n=7)           | (n=7)           | (n=6)           | (n=7)           |
| Cholesterol, mmol/L | 1.67 ± 0.16     | 9.65 ± 1.26*    | 1.92 ± 0.16     | 8.34 ± 0.54†    |
| LDL, mmol/L      | 0.58 ± 0.12     | 7.24 ± 1.06*    | 1.08 ± 0.13     | 6.76 ± 0.64†    |
| MAP, mm Hg       | 101.8 ± 4.4     | 105.0 ± 4.0     | 121.6 ± 9.0*    | 121.9 ± 4.3*    |
| PRA, ng·mL⁻¹·h⁻¹ | 0.51 ± 0.31     | 0.36 ± 0.08     | 0.28 ± 0.06     | 0.75 ± 0.41     |
| Creatinine, μmol/L | 129.9 ± 6.2     | 160.6 ± 7*      | 165 ± 11.5*     | 182.1 ± 21.2*   |

**Redox status: plasma**

- Vitamin C, μmol/L | 94 ± 2.54 | 85 ± 2.9* | 87.7 ± 1.44* | 76.7 ± 2.5†† |
- Vitamin E, μmol/L | 60.5 ± 2.8 | 51.5 ± 1.9* | 62.8 ± 1.3† | 44.3 ± 2.1†† |
- LDH lag time, min  | 83.7 ± 3.0  | 73.8 ± 2.5* | 82.6 ± 1.4† | 68.8 ± 1.9†† |
- LDH MDA, nmol/L·mg⁻¹ | 6.6 ± 0.4 | 8.5 ± 0.2* | 7.1 ± 0.2‡ | 9.2 ± 0.2†† |
- LDH REM, mm        | 10.9 ± 0.3  | 12.3 ± 0.3* | 10.5 ± 0.3† | 12.8 ± 0.3†† |

**Redox status: tissue, μU/mg protein**

- Glutathione peroxidase | 86.5 ± 2.5 | 86.6 ± 2.2 | 66.1 ± 1.7†‡ | 59.7 ± 2.1†† |
- Catalase             | 23.5 ± 1.3  | 21.6 ± 1.2 | 16.9 ± 1.0†| 14.5 ± 0.6†† |
- Mn-SOD               | 3.2 ± 0.1   | 3.2 ± 0.1 | 2.6 ± 0.1* | 1.9 ± 0.1†† |
- CuZn-SOD             | 8.1 ± 0.2   | 8.0 ± 0.2 | 7.3 ± 0.1* | 6.7 ± 0.2†† |

**MAP** indicates mean arterial pressure; MDA, malondialdehyde; and REM, relative electrophoretic mobility. Values are mean ± SEM.

*P < 0.05 vs normal; †P < 0.05 vs RAS; ‡P < 0.05 vs HC.

### Table 2. Basal Single-Kidney Hemodynamics and Function in Normal, HC, RAS, and HC + RAS Pigs

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|-----------------|-----------------|-----------------|-----------------|
|                 | Normal          | HC              | RAS             | HC + RAS        |
|                 | (n=7)           | (n=7)           | (n=6)           | (n=7)           |
| Volume, mL      |                 |                 |                 |                 |
| Cortex          | 100.4 ± 6.2     | 95.1 ± 5.7      | 55.2 ± 15.3†    | 75.2 ± 4.1†     |
| Medulla         | 45.4 ± 2.3      | 41.8 ± 6.9      | 24.7 ± 6.6‡     | 30.3 ± 3.3†     |
| Renal blood flow, mL/min | 534.0 ± 49.1 | 499.5 ± 44.5 | 266.9 ± 99.9* | 364.7 ± 54.5* |
| Glomerular filtration rate, mL/min | 70.8 ± 4.3 | 60.3 ± 3.1 | 31.8 ± 11.1* | 50.4 ± 3.5* |
| Perfusion, mL·min⁻¹·g⁻¹ |                 |                 |                 |                 |
| Cortex          | 4.11 ± 0.33     | 4.36 ± 0.43     | 3.27 ± 0.62†    | 3.31 ± 0.33†    |
| Medulla         | 2.63 ± 0.33     | 3.13 ± 0.66     | 2.91 ± 0.17     | 2.34 ± 0.34††   |
| Papilla         | 2.38 ± 0.33     | 2.59 ± 0.55     | 1.99 ± 0.43     | 2.42 ± 0.76     |

Values are mean ± SEM.

*P < 0.05 vs normal; †P < 0.05 vs HC, ‡P < 0.05 vs RAS.
In normal animals, SNP increased RBF (to 641.5 ± 58.8 mL/min, \( P<0.03 \), Figure 1d) and cortical and medullary perfusion. In contrast, in HC, RAS, and HC+RAS, RBF (Figure 1d) and regional perfusion remained unchanged. GFR and ITC were unaltered in all groups (\( P>0.05 \)).

**Redox Status**

HC animals showed a decrease in circulating vitamins C and E, whereas RAS pigs had lower levels of vitamin C, and in HC+RAS, both vitamin levels were lowered further compared with all other groups (Table 1). In HC but not RAS, systemic LDL showed increased oxidizability (shortened lag time and increased LDL-malondialdehyde and LDL-relative electrophoretic mobility). Nevertheless, this was accentuated in HC+RAS compared with HC alone (Table 1). Tissue activities of radical-scavenger enzymes were significantly lower in RAS than with normal but were unchanged in HC. Notably, in stenotic HC+RAS kidneys, the decrease in scavenging activity was significantly greater than in each group alone (Table 1).

**Renal Histology**

Compared with normal, HC kidneys showed mild and focal interstitial fibrosis with associated tubular atrophy. RAS kidneys showed more extensive regions of focal interstitial fibrosis and tubular atrophy, with patchy lymphocytic infiltrates localized to regions of tubular atrophy and isolated sclerotic glomeruli. HC+RAS kidneys, however, showed multifocal interstitial fibrosis with associated tubular atrophy. Globally, sclerotic glomeruli were identified, primarily within the regions of interstitial fibrosis and tubular atrophy (Figure 2a). Focal lymphocytic infiltrates were identified within the expanded interstitial regions. Mild and focal perivascular fibrosis was noted (Figure 2b). The media-to-lumen ratio and glomerular score were increased in all stenotic kidneys (Table 3).

These findings were accompanied in HC by enhanced glomerular, vascular, and/or tubular immunostaining of trichrome, iNOS, nitrotyrosine, TGF-\( \beta \), and NF-\( \kappa B \), which were further increased in RAS and HC+RAS compared with HC (Table 3, Figures 2 and 3). Moreover, trichrome, iNOS, nitrotyrosine, and NF-\( \kappa B \) immunostaining was further significantly increased in HC+RAS compared with all other groups. No differences in eNOS expression were observed (Figure 3a), although it tended to be decreased in HC.

All measures of oxidative stress, proximal and Henle’s loop ITC, and immunostaining of trichrome, iNOS, nitrotyrosine, and NF-\( \kappa B \) showed significant interactive effects between HC and RAS (ANOVA, \( P<0.05 \)).

**Discussion**

This study demonstrates that coexistence of early HC and hypoperfusion is associated with greater renal functional and structural impairment, especially in the tubular and glomerular systems, compared with each condition alone. This was accompanied by pronounced increases in both systemic and tissue oxidative stress and proinflammatory and early pro-growth changes in the stenotic kidney, which might be involved in progression of renal injury that characterizes atherosclerotic RAS.
Atherosclerotic RAS is increasingly being identified in patients with ESRD, and clinical studies consistently demonstrate unfavorable outcomes compared with other causes of RAS, suggesting that additional mechanisms contribute to the deleterious effect of hypoperfusion on the kidney and augment renal injury. Atherosclerosis or HC and renal hypoperfusion might interact in several common pathophysiological pathways to disrupt the balance among intrarenal vasoactive factors that regulate vascular tone and tissue growth. Acceleration of vascular, tubular, and glomerular injury may be mediated by inhibition of NO-mediated vasodilatation, activation of angiotensin II, increased generation of ROSs, LDL oxidation, and modulation of cell growth and proliferation. This cascade, which is shared by many forms of renal disease, may lead to vasoconstriction, glomerulosclerosis, and fibrosis and impair renal function and structure.

The EBCT technique allowed unique measurement of concomitant hemodynamics and function of the intact single kidney distal to RAS. The degree of stenosis, which was slightly (albeit not significantly) greater in RAS than HC+RAS, might have resulted in somewhat but insignificantly lower renal volume, RBF, and GFR in RAS, all of which were decreased in both groups compared with normal and HC. Interestingly, basal ITC and GFR and tubular responses to challenge were significantly more impaired in HC+RAS, indicating that concurrent HC promoted functional renal injury. This was particularly obvious in the renal tubules, which showed in vivo decreased basal filtrate concentration capacity, a measure of intrinsic renal damage, and blunted response to challenge. GFR failure to respond to challenge may also indicate an early pathophysiological mechanism that can accompany altered tubular reabsorption. Conversely, renovascular dysfunction was not substantially augmented by concurrent HC. These in vivo findings were supported by morphological examination of the stenotic HC+RAS kidney, which revealed a marked increase in tubulointerstitial accompanied by less pronounced glomerular injury and milder vascular changes. Similarly, alterations in iNOS, NF-kB, and TGF-β expression, suggestive of inflammation and fibrosis, were localized principally to the tubulointerstitial and, to a lesser degree, glomerular and vascular compartments.

### Table 3. Morphological Evaluation and Immunostaining in Normal, HC, RAS, and HC+RAS Kidneys

<table>
<thead>
<tr>
<th></th>
<th>Normal (n=7)</th>
<th>HC (n=7)</th>
<th>RAS (n=6)</th>
<th>HC+RAS (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glomerulosclerosis</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>4.3±0.8*</td>
<td>4.7±1.2*†</td>
</tr>
<tr>
<td>Media-lumen ratio</td>
<td>0.24±0.00</td>
<td>0.26±0.01*</td>
<td>0.61±0.07†</td>
<td>0.64±0.04†</td>
</tr>
<tr>
<td>Trichrome</td>
<td>3.0±0.2</td>
<td>6.6±0.8*</td>
<td>11.7±0.8†</td>
<td>15.6±0.6†‡</td>
</tr>
<tr>
<td>iNOS</td>
<td>2.6±0.7</td>
<td>9.0±0.9*</td>
<td>15.2±0.9†</td>
<td>17.9±0.4†‡</td>
</tr>
<tr>
<td>Nitrotyrosine</td>
<td>7.6±0.3</td>
<td>8.7±0.2*</td>
<td>9.2±0.3†</td>
<td>10.4±0.5†‡</td>
</tr>
<tr>
<td>TGF-β</td>
<td>5.4±0.4</td>
<td>7.1±0.3*</td>
<td>9.5±0.3†</td>
<td>9.3±0.2†</td>
</tr>
<tr>
<td>NF-kB</td>
<td>0.3±0.1</td>
<td>1.4±0.4*</td>
<td>3.6±1.1†</td>
<td>6.4±0.8†‡</td>
</tr>
</tbody>
</table>

Values are % of renal area, mean±SEM.

*P<0.05 vs normal; †P<0.05 vs HC; ‡P<0.05 vs RAS.
This concordance of in vivo and in vitro findings may also suggest a potential role for ITC as an index of tubular integrity and function. The renal tubules are distinctly vulnerable to renal circulatory compromise and hypoxic injury, and renal susceptibility to hypoperfusion is aggravated by insults that increase medullary vulnerability to hypoxia, especially models combining multiple insults. Indeed, medullary perfusion was significantly decreased in HC+RAS. In chronic conditions, intrarenal hypoperfusion injury progresses to encompass the entire kidney. The proximal tubule, and particularly its S3 segment, is also highly susceptible to injury, and the decreased ITC observed in both the proximal tubule and Henle’s loop in HC+RAS might have represented early functional injury in these tubular segments.

Furthermore, a progressive and marked increase in iNOS expression along the nephron paralleled the increasing severity of renal disease in the HC, RAS, and HC+RAS kidneys. The constitutive tubular expression of iNOS is enhanced in renal disease by cytokines, increased ROS activity, and peroxynitrite. Increased iNOS expression may mediate hypoxic tubular injury or alternatively might conceivably reflect a compensatory mechanism to decrease oxygen consumption during renal ischemia. Its natriuretic effects may explain the enhanced ITC response observed in HC, whereas in RAS, ITC probably increased secondary to angiotensin II–driven proximal fluid resorption that outweighed the moderately elevated iNOS expression. In HC+RAS, however, tubular atrophy in association with pronounced tubular iNOS expression may have attenuated basal and challenged ITC.

In contrast to iNOS-derived tubular NO, renovascular dysfunction, demonstrated by similarly blunted RBF responses to both endothelium-dependent and -independent vasodilators in HC, RAS, and HC+RAS, implied decreased bioavailability and/or responsiveness to vascular NO. This impairment, in view of unaltered eNOS expression, may result from altered eNOS activity, increased sensitivity to vasoconstrictors, or quenching of NO by ROSs, indicated in the present study by significant peroxynitrite formation. Furthermore, increased iNOS activity may also inhibit eNOS and lead to renal vasoconstriction and reduction in GFR.

The functional alterations in HC+RAS were accompanied by marked tissue injury, increased LDL oxidizability, and decreased tissue activities of radical scavengers, denoting an amplified increase in systemic and tissue oxidative stress. Renal histology revealed considerable tubulointerstitial and, to a lesser degree, glomerular and vascular injury in the HC+RAS kidneys, in association with increased expression of NF-κB, a transcription factor that regulates expression of numerous genes involved in inflammation and cell proliferation. Notably, immunoreactivity of TGF-β, which participates in renal injury and progression to ESRD, was similarly enhanced in RAS and HC+RAS, suggesting that additional progrowth mechanisms probably played a role in tissue fibrosis in HC+RAS.

Indeed, in atherosclerotic RAS patients, additional risk factors, longer duration, and chronicity could influence renal damage, and vascular injury probably progresses, because we have shown that their decrease in cortical perfusion exceeds the degree of stenosis. In the present study, media-to-lumen ratio was comparably increased in RAS and HC+RAS and glomerulosclerosis was minimal, suggesting that the HC+RAS model mimics a very early stage of ischemic...
nephropathy. This study used a swine model, the renal anatomy and physiology of which resemble those of humans. Diet-induced HC, a surrogate of early atherosclerosis, and our RAS model possess many characteristics of human conditions. Therefore, the HC+RAS model provides an important opportunity to explore relevant pathophysiological mechanisms triggered early in this disease process.

In summary, the present study demonstrates substantial accentuation of the impact of RAS and augmentation of early functional and tissue injury in HC+RAS distal to the stenosis in vivo and in vitro. These observations suggest a cross talk between hyperperfusion and atherosclerosis to interactively increase oxidative stress, inflammation, and tubular injury in the stenotic kidney, perpetuating a vicious circle of potentially irreversible injury. These deleterious alterations may account for the increased propensity for ESRD observed in this disease.

Acknowledgments

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References

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