Pathways of Renal Fibrosis and Modulation of Matrix Turnover in Experimental Hypercholesterolemia


Abstract—Dyslipidemia often accompanies and accelerates renal disease, partly by promoting fibrosis. However, the mechanisms mediating this effect are unclear. We hypothesized that hypercholesterolemia modulates several interlinked pathways that promote deposition and blunt degradation of extracellular matrix, and that these could be manipulated by reversal of hypercholesterolemia. Fourteen pigs were fed a 16-week 2% high-cholesterol diet (HC-HC; n=7) or normal diet (n=7), whereas in 7 others, a 10-week HC was followed by a 6-week normal diet (HC-N). Renal endothelial function was assessed in vivo with electron-beam computed tomography, and renal tissue was then studied ex vivo using Western blot, real-time quantitative polymerase chain reaction, gelatin zymography, and immunostaining. HC-HC kidneys showed endothelial dysfunction, accompanied by increased intrarenal oxidative stress, inflammation, activation of the endothelin and transforming-growth-factor-β systems, and decreased matrix metalloproteinase expression and activity. Accordingly, HC-HC kidneys showed increased collagen IV expression and fibrosis. A lipid-lowering dietary intervention reversed most of these changes. In conclusion, this study indicates that renal fibrosis in early atherosclerosis is a result of a simultaneous increase in extracellular matrix deposition and blunted matrix metalloproteinase-mediated degradation, overall promoting perivascular and tubulointerstitial fibrosis. Notably, many of these pathways may be reversible in hypercholesterolemia, and crucial targets could potentially be identified for early interventions to preserve the kidney. (Hypertension. 2005;46:772-779.)

Key Words: kidney ■ hypercholesterolemia ■ fibrosis ■ remodeling ■ oxidative stress

Hypercholesterolemia is a common cardiovascular risk factor. Approximately 50% of the middle-aged adult population has total cholesterol above desirable levels,1 a strong, independent predictor of progression of atherosclerosis.2 Dyslipidemia also often accompanies and aggravates early and advanced renal disease.3,4 We have previously shown in a pig model that a 10- to 12-week diet-induced hypercholesterolemia, a risk factor for early atherosclerosis, elicited renal dysfunction, inflammation, and fibrosis, partly mediated by increased oxidative stress.5–8 Increased oxidative stress may contribute to renal damage in hypercholesterolemia and atherosclerosis by virtue of augmented generation of reactive oxygen species (ROS) and oxidation of LDLs,8–12 which may induce endothelial dysfunction and activate redox-sensitive growth factors and cytokines. In addition, lipid accumulation or cellular damage can promote renal dysfunction, glomerular injury, and interstitial fibrosis.13,14

A dynamic and complex process in which tissue growth is counterbalanced by degradation and removal preserves the normal structure of the kidney and may be partly modulated by endothelium-derived factors. Kidney disease and normal renal development are characterized by a high rate of extracellular matrix (ECM) turnover. Several fibrogenic factors are implicated in this process, such as transforming growth factor-β (TGF-β), angiotensin II, and endothelin-1 (ET-1).15 whereas the major regulators of renal ECM degradation are the matrix metalloproteinases (MMP) family.16 We observed previously that in the stenotic kidney, concurrent hypercholesterolemia amplifies renal injury and scarring,5,12 but the pathways by which dyslipidemia modulates renal fibrosis have not been completely defined. This study was designed to test the hypothesis that hypercholesterolemia not only promotes fibrogenic activity but also downregulates renal MMP, thereby blunting ECM degradation and facilitating renal scarring. Further, these mechanisms could be manipulated by a dietary lipid-lowering intervention.

Methods

The institutional animal care and use committee approved all procedures. Twenty-one domestic cross-bred pigs (65 to 75 kg) were studied after 16 weeks of observation. The pig model was selected because of the similarity between swine and human cardiovascular and renal physiology, lipid profile, and body size. In 14 pigs, early atherosclerosis was simulated by feeding them with a 2% high-cholesterol (HC) diet (TD-93296; Harlan Teklad).6–9 Of these, 7 ani-
mals were fed for 10 weeks with the HC diet and then changed over to a normal pig chow for the remaining 6 weeks (HC-N; n = 7), whereas in the other 7, the HC diet was sustained for the entire 16 weeks (HC-HC; n = 7). We have previously shown that a 10- to 12-week HC diet in the swine resulted in endothelial dysfunction and activation of injurious mechanisms in the absence of obstructive atherosclerotic plaques in different vascular beds (eg, aorta, renal, or coronary arteries), similar to humans with early atherosclerosis. An additional group of pigs fed with a normal diet for 16 weeks was used as controls (normal; n = 7). Venous blood samples were collected from all pigs after 10 and 16 weeks of diet, and in vivo and in vitro studies were performed at 16 weeks.

On the day of in vivo studies, each animal was anesthetized with intramuscular telazol (5 mg/kg) and xylazine (2 mg/kg), intubated, and mechanically ventilated with room air. Anesthesia was maintained with a mixture of ketamine (0.2 mg/kg per minute) and xylazine (0.03 mg/kg per minute) in normal saline and administered via an ear vein cannula (0.05 mL/kg per minute). Catheters were placed under fluoroscopic guidance in the aorta and right atrium, and electron-beam computed tomography (EBCT; C-150; Imatron) flow studies were then performed in vivo under baseline conditions and repeated during aortic suprarenal infusion of acetylcholine (Ach) to test renal endothelial function, as detailed previously. PET-A and ET-B receptors and NAD(P)H-oxidase subunit gp91phox were assessed in 20 to 30 randomly selected intrarenal and intramycardial vessels, as described previously.

After completion of the studies, pigs were killed with an intravenous injection of Sleepaway (sodium pentobarbital; 100 mg/kg; Ft Dodge Laboratories). Kidneys were removed using a retroperitoneal approach, snap-frozen in liquid nitrogen, and stored at −80 °C or preserved in formalin. Total prostaglandin F2α-isoprostanes (enzyme immunooassay; Cayman Chemical Company), superoxide dismutase (SOD) activity (spectrophotometry; Cayman Chemical Company), and circulating oxidized LDL (Ox-LDL) levels (ELISA; Mercodia) were measured.

In vitro studies were then performed to evaluate redox status and renal proinflammatory and profibrotic activity. Using Western blot and real-time quantitative RT-PCR, renal protein or mRNA expression of the endothelial NO synthase (eNOS), prepro–ET-1, and its ET-A and ET-B receptors and NAD(P)H-oxidase subunit gp91phox and p22phox were measured. To assess the expression of fibrogenic as well as antiﬁbrotic factors, we measured the expression of the profibrotic TGF-β and its downstream mediators Smad-2/3, and Smad-4, plasminogen activator inhibitor type 1 (PAI-1), and tissue inhibitor of metalloproteinase-1 (TIMP-1) and TIMP-2. To assess matrix degradation, the expression and activity of MMP-2 and MMP-9 and expression of membrane type 1 (MT1)–MMP, thrombospondin 1/2 (TSP 1/2), and collagen IV was quantified. In addition, using deparaffinized 5-μm-thick mid-hilar cross-sections, renal morphology (hematoxylin/eosin), inflammation (ED-1 and CD-3), and fibrosis (trichrome, collagen IV) were also evaluated. Finally, myocardial expression of MMP-2 and MMP-9 and trichrome staining were also determined.

Renal Protein Expression

Western Blotting

Standard blotting protocols were performed in homogenized renal tissue (mainly cortical) as described previously. For details, see the online supplement, available at http://www.hypertensionaha.org.

Real-Time Quantitative PCR

Total RNA was isolated from kidneys using the TRIZOL (InvitrogenTM) method. cDNA was synthesized using InvitrogenTM SuperScriptTM first-strand synthesis kit as we described recently. To investigate the expression of prepro–ET-1, –ET-A, and –ET-B receptors, and MMP-2, MMP-9, TIMP-1, and TIMP-2 mRNA, RT-PCR (DNA engine OPTICON; MJ Research) was performed using SYBR Green JumpStartTM Taq ReadyMixTM kit (Sigma). For details, see the online supplement.

Immunohistochemistry for CD-3 and ED-1 was performed on deparaffinated renal tissue, and for collagen IV, in frozen cross-sections.

Gelatin Zymography

MMP-2 and MMP-9 were assayed for gelatinolytic activity by means of gelatin zymography following standard procedures. For details, see the online supplement.

Data Analysis

Manually traced regions of interest were selected in EBCT images in the aorta, renal cortex, medulla, and papilla, and their densities sampled. Single-kidney RBF, GFR, and renal regional perfusion (ml/g per minute per cc tissue) before and after Ach infusion were calculated using previously validated methods.

Histology

Mid-hilar cross-sections of the kidney (including cortical and medullary regions) and transmural left ventricular myocardium (1 per animal) were examined as vascular media-to-lumen ratio was evaluated in 20 to 30 randomly selected intrarenal and intramyocardial vessels, as described previously.

Statistical Analysis

Results are expressed as mean ± SEM. Comparisons within groups were performed using paired Student t test, and among groups using ANOVA, with the Bonferroni correction for multiple comparisons, followed by unpaired Student t test. In case of non-normal distribution, nonparametric (Wilcoxon/Kruskall–Wallis) tests were applied to compare among the groups. Statistical significance was accepted for P ≤ 0.05.

Results

After 10 weeks of HC diet, total and LDL cholesterol as well as Ox-LDL levels were significantly and similarly elevated in HC-HC and HC-N groups compared with the normal group, whereas SOD activity was significantly lower (Table). All these were normalized in HC-N after changing to 6 weeks of normal diet but remained elevated in HC-HC. HDL cholesterol was similarly elevated in HC-HC and HC-N compared with controls at 10 weeks, as we have shown previously, and remained elevated in HC-HC at 16 weeks. In addition, after 16 weeks, total isoprostanes were significantly elevated only in HC-HC; whereas mean arterial pressure was not different among the groups (Table). We have previously shown in our model that a 12-week HC diet consistently did not increase blood pressure either.

Renal Endothelial Function and Oxidative Stress

Basal single-kidney volume, RBF, GFR, and regional perfusion measured after completion of the protocol were similar among the groups (Table). Infusion of Ach in normal and HC-N animals was associated with a similarly significant increase in RBF, GFR (Figure 1; P ≤ 0.05 versus baseline), and cortical perfusion (to 5.5 ± 0.5 and 5.7 ± 0.5 mL/g per
minute per cc, respectively; \( P \leq 0.05 \) versus baseline), whereas medullary and papillary perfusion were significantly elevated only in normal animals \( (P < 0.05) \). However, this response was completely blunted in the HC-HC group, in which Ach did not increase RBF, GFR (Figure 1), or any regional perfusion compared with baseline.

Accompanying the blunted renal function in HC-HC, the expression of NAD(P)H-oxidase (subunits gp91phox and p22phox) were increased, whereas eNOS was attenuated compared with normal animals (Figure 2a), suggesting increased potential for superoxide generation and decreased NO bioavailability. Furthermore, HC-HC showed a significant increase in renal mRNA expression of prepro–ET-1 and –ET-A receptors, suggesting upregulation of the endogenous ET system (Figure 2b).

### Renal Morphology and Fibrogenic Factors

HC-HC kidneys showed increased perivascular and tubulointerstitial accumulation of CD-31 and ED-1 inflammatory cells, which was more evident in the cortex (Figure 3a and 3b). In addition, HC-HC also showed a significant increase in cortical perivascular and tubulointerstitial fibrosis (collagen IV and trichrome; Figure 3c and 3d) and increased interlobular and arcuate artery media-to-lumen ratio (Figure 3c, bottom). Glomerulosclerosis was not observed in any group.

### Extracellular Matrix

Renal cortical expression of the profibrotic TGF-\( \beta \), its mediators Smad-2/3 and Smad-4, and collagen IV were all significantly increased in HC-HC, whereas PAI-1 remained unchanged (Figure 4). In contrast, the expression of MT1-

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**Table: Systemic Characteristics and Basal Single-Kidney Hemodynamics (mean±SEM)**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal ( n=7 )</th>
<th>HC-HC ( n=7 )</th>
<th>HC-N ( n=7 )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total cholesterol (mmol/L)</strong></td>
<td></td>
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<tr>
<td>10 weeks</td>
<td>1.9±0.2</td>
<td>9.9±2.2*</td>
<td>11.4±3.7*</td>
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<tr>
<td>16 weeks</td>
<td>1.8±0.4</td>
<td>10.4±1.6*</td>
<td>2.0±0.1</td>
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<tr>
<td><strong>LDL cholesterol (mmol/L)</strong></td>
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<tr>
<td>10 weeks</td>
<td>0.9±0.2</td>
<td>7.4±2.2*</td>
<td>8.6±3.3*</td>
</tr>
<tr>
<td>16 weeks</td>
<td>1.0±0.3</td>
<td>7.4±1.5*</td>
<td>0.9±0.1</td>
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<tr>
<td><strong>Ox-LDL (U/mL)</strong></td>
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<tr>
<td>10 weeks</td>
<td>8.6±0.7</td>
<td>19.6±3.1*</td>
<td>22.3±3.9*</td>
</tr>
<tr>
<td>16 weeks</td>
<td>8.5±0.6</td>
<td>23.6±3.3*</td>
<td>9.8±0.7</td>
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<tr>
<td><strong>HDL (mmol/L)</strong></td>
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<tr>
<td>10 weeks</td>
<td>0.86±0.1</td>
<td>1.3±0.2*</td>
<td>1.4±0.3*</td>
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<tr>
<td>16 weeks</td>
<td>0.84±0.1</td>
<td>1.4±0.4*</td>
<td>0.8±0.1</td>
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<tr>
<td><strong>Mean arterial pressure (mm Hg)</strong></td>
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<td></td>
<td>111.8±4.4</td>
<td>117.1±4.1</td>
<td>117.9±8.9</td>
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<tr>
<td><strong>Plasma SOD activity (U/mL)</strong></td>
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<tr>
<td>10 weeks</td>
<td>3.4±0.4</td>
<td>1.9±0.3*</td>
<td>2.2±0.4*</td>
</tr>
<tr>
<td>16 weeks</td>
<td>3.5±0.3</td>
<td>2.4±0.2*</td>
<td>4.1±0.5</td>
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<tr>
<td><strong>Total isoprostanes (pg/mL)</strong></td>
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<td></td>
<td>98.3±2.5</td>
<td>178.0±0.0*</td>
<td>88.0±8.4</td>
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<tr>
<td><strong>Renal volume (cc)</strong></td>
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<td></td>
<td>148.7±13.7</td>
<td>136.2±7.2</td>
<td>138.7±4.1</td>
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<tr>
<td><strong>RBF (mL/min)</strong></td>
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<td></td>
<td>613.9±71.3</td>
<td>511.4±50.4</td>
<td>631.2±59.1</td>
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<tr>
<td><strong>GFR (mL/min)</strong></td>
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<td></td>
<td>81.8±9.4</td>
<td>68.3±5.5</td>
<td>71.9±5.6</td>
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<tr>
<td><strong>Perfusion (mL/min/cc)</strong></td>
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<tr>
<td>Cortex</td>
<td>4.4±0.2</td>
<td>3.9±0.4</td>
<td>4.6±0.5</td>
</tr>
<tr>
<td>Medulla</td>
<td>3.0±0.3</td>
<td>2.8±0.4</td>
<td>3.3±0.4</td>
</tr>
</tbody>
</table>

Measurements were obtained after a 16-week diet, unless otherwise indicated. \( *P < 0.05 \) vs normal.

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**Figure 1.** RBF (a) and GFR (b) at baseline (gray bars) and in response to Ach (mL/min; black bars) in normal, hypercholesterolemic (HC-HC), and HC pigs after lipid lowering (HC-N). Reversal of the diet normalized the blunted responses in HC-HC. \( *P < 0.05 \) vs baseline.
MMP, MMP-2, and MMP-9 protein was attenuated in HC-HC, attended by elevated expression of the MMP inhibitors TIMP-1, TIMP-2, and TSP 1/2, suggesting blunted ECM degradation (Figure 4). Notably, HC-HC kidneys also showed decreased activity of MMP-2 and MMP-9, although MMP and TIMP mRNA expression was not different among the groups (Figure 5).

For comparison purposes, we also investigated the expression of MMPs in the heart of the same animals. Interestingly, MMP-9 was unaltered, but MMP-2 showed a strong trend to increase in HC-HC compared with normal (P=0.08; supplemental Figure, available online at http://www.hypertensionaha.org), which was accompanied by significant myocardial interstitial fibrosis but preserved media-to-lumen ratio (P=NS).

**Lipid-Lowering Intervention**
After the 6-week normal diet, the expression of gp91phox, p22phox, eNOS (Figure 2), TGF-β/Smads, MMPs/TIMP, and TSP 1/2 in HC-N were not different from normal (Figures 4 and 5). The expression of prepro–ET-1 and –ET-A receptors, suggesting a decreased potential for vasoconstriction. *P<0.05 vs normal or HC-N; †P<0.05 vs HC-HC.
renal inflammation and fibrosis in HC-N diminished compared with HC-HC (Figure 3).

**Discussion**

This study suggests that renal fibrosis in the early stage of atherosclerosis is the result of simultaneous modulation of a number of injury pathways. HC induced renal endothelial dysfunction, upregulated the intrarenal endothelin, TIMP, and TGF-β systems, and blunted MMPs, overall favoring renal scarring by facilitating ECM deposition and blunting matrix degradation. Importantly, the current study also shows that dietary intervention may partly revert the deleterious effects of hypercholesterolemia and decrease renal functional and structural injury.

Considerable effort has been made in the past years to elucidate the mechanisms of chronic kidney disease, an increasingly common condition often leading to destruction of the kidney. Lipid abnormalities have been increasingly recognized as a pivotal risk factor for renal disease progression. Notably, even a short-term exposure to cholesterol may impair vascular endothelial function, which in turn may facilitate tissue injury by favoring vasoconstriction, increased activity of mitogens such as ET-1, and decreased buffering effects of the endogenous vasodilator and antimitotic NO, which normally curtails inflammation and fibrosis. Indeed, we have previously shown that a 10- to 12-week HC diet was sufficient to significantly impair renovascular function and induce intrarenal inflammation, vascular remodeling, and perivascular and tubulointerstitial fibrosis. The current study extends our previous observations by exploring the downstream mechanisms that mediate renal injury in hypercholesterolemia. We observed that hypercholesterolemia modulates pathways involved in the dynamic process of tissue remodeling, which includes ECM synthesis, deposition, and removal.

A prominent factor likely involved in renal injury in the hypercholesterolemic kidney is Ox-LDL. We have previously shown that diet-induced hypercholesterolemia increased renal expression of the lectin-like specific receptor for ox-LDL (LOX-1), the specific receptor for Ox-LDL uptake, which may also be regulated by TGF-β and endothelin. The current study further shows increased circulating levels of Ox-LDL in HC-HC, which may have resulted from the concurrent increases in systemic oxidative stress (suggested by the elevated isoprostanes and blunted ROS scavenging) and in the availability of LDL achieved by this dietary regimen. Glomerular mesangial, endothelial, and vascular smooth muscle cells can uptake oxidized as well as nonoxidized lipids. Native LDL and Ox-LDL can harm the kidney either directly, by deposition of lipids, or indirectly, by stimulating the generation of ROS. These mechanisms may lead to endothelial dysfunction as well as glomerular injury by inducing formation of foam cells that are associated with interstitial damage and later glomerulosclerosis. In addition, ROS and Ox-LDL may upregulate in the hypercholesterolemic kidney key factors such as nuclear factor...
κB, which participates in inflammation, atherogenesis, and cell proliferation. Moreover, Ox-LDL also promotes fibrosis by stimulating synthesis and expression of TGF-β. The redox-sensitive TGF-β regulates many fundamental biological processes and is a key mediator in chronic kidney disease. We observed previously in early atherosclerosis increased tubular and glomerular TGF-β expression, which could be manipulated by pharmacological intervention. The current study further demonstrates that increased TGF-β expression in the hypercholesterolemic kidney is accompanied by upregulation and activation of its Smad effectors, underscoring fibrogenic activity. Phosphorylation of the TGF-β receptors activates its intracellular signaling effectors, the Smad proteins, which then translocate into the nucleus to regulate transcription. Receptor-regulated Smad-2 and Smad-3 are phosphorylated by the TGF-β receptor, a process that is indispensable to link to Smad-4 (cooperative Smad) and therefore initiates recruitment of transcriptional cofactors involved in cell proliferation and tissue growth.

In addition, TGF-β is also a potent inhibitor of ECM degradation. Indeed, it stimulates production of other profibrotic factors such as TIMPs, which induce cell proliferation directly and via inhibitory effects on MMPs. Hence, the increase in TIMP-1 and TIMP-2 protein expression was accompanied by downregulation of the expression and activity of MMP-2, MT1-MMP (involved in MMP-2 activation), and MMP-9. MMP-2 and MMP-9 are capable of digesting denatured and native collagen IV, which is often produced in the damaged kidney, as we observed in HC-HC animals. The MMPs have a key role preserving the damaged kidney. They can be regulated at the level of gene expression, activation of proenzymes, or inhibition by complexing with their specific TIMPs, and can be modulated by cytokines, hormones, and growth factors. Interestingly, MMP renal mRNA expression remained unaltered, possibly suggesting post-transcriptional regulation in HC-HC. The increased TSP in HC-HC kidneys may have contributed to this process because TSP-1 and TSP-2 favor degradation of MMP-2 and MMP-9 and can activate TGF-β. Overall, the blunted MMPs expression and activity may be part of an imbalance favoring ECM production and accumulation in the hypercholesterolemic kidney and may have ultimately been responsible for the fibrosis and microvascular wall thickening observed in this group. In contrast, we and others have previously shown that in coronary arteries and atherosclerotic plaques, early and advanced atherosclerosis were associated with increased MMP expression. The current study extends these observations and showed that myocardial fibrosis was attended by a tendency for increased myocardial expression of MMP-2 in HC-HC animals, which contrasts with its decreased expression in the kidneys. These observations suggest that myocardial fibrosis might be mediated by different mechanisms, and that the downregulation of MMP in the atherosclerotic kidney may be relatively unique to this organ.

Recent experimental studies have underscored the potential to revert renal structural damage in animal models of renal injury such as aging, diabetic nephropathy, and subtotal nephrectomy. In these studies, regression of renal lesions was achieved using targeted pharmacological interventions such as ET-A blockers or angiotensin-converting enzyme inhibitors or by increasing leptin expression and levels, suggesting the involvement of these systems in the mechanisms of renal injury. Our study suggested that in hypercholesterolemia, renal injury may also be prevented or reverted by controlling dyslipidemia. Indeed, we observed that after a lipid-lowering dietary intervention, renal hemodynamic responses to the prototypical endothelium-dependent vasodilator Ach in vivo were normalized, likely as a result of restoration of eNOS expression, downregulation of ET, and increased NO bioavailability. A decrease in ROS abundance

Figure 5. Renal enzymographic activity (top) and mRNA expression (bottom) of MMP-9 and MMP-2 in normal, hypercholesterolemic (HC-HC), and hypercholesterolemic pigs after lipid lowering (HC-N). HC-HC kidneys showed decreased MMP-9 and MMP-2 activities (but not mRNA expression), which were improved after lipid-lowering intervention.
(denoted by reduced gp91phox renal expression, increased SOD activity, and reduced isoprostanes levels) may have also improved endothelial function and attenuated renal injury in HC-N. Furthermore, the decrease in interstitial and vascular fibrosis may have also contributed to improved vascular function. Of note, only cortical but neither medullary nor papillary perfusion was improved. This may imply that circulating and locally acting vasoactive agents (such as ET, the expression of which remained high) may exert a differential regulation of the renal vasculature and may reflect the sensitivity of these renal zones to noxious milieu. Furthermore, it is possible that additional mechanisms, such as the renin-angiotensin system, require pharmacological intervention for more complete regression of renal injury.

Perspectives

The current study implies a distinct pathway of renal injury in early atherosclerosis, which seems to be the result of a coordinated increase in ECM production and blunted degradation, overall favoring renal scarring. Longer duration and more advanced atherosclerosis may perpetuate these mechanisms and accelerate deterioration of renal function. Nevertheless, reversal of the HC diet in swine attenuated renal inflammation, normalized the expression of most fibrogenic factors, and regressed vascular and renal fibrosis. Thus, many of these pathways may be reversible, at least at the early phase, and therefore provide rationale for the use of lipid-lowering strategies to preserve the kidney in hypercholesterolemia.

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