Role of Renal Cortical Neovascularization in Experimental Hypercholesterolemia

Alejandro R. Chade, James D. Krier, Offer Galili, Amir Lerman, Lilach O. Lerman

Abstract—Hypercholesterolemia induces renal inflammation and neovascularization, associated with renal endothelial dysfunction and injury. Neovascularization might conceivably represent a defense mechanism to sustain renal perfusion. Therefore, the present study was designed to test the hypothesis that preventing neovascularization using thalidomide, a potent anti-inflammatory and antiangiogenic agent, would impair basal renal hemodynamics in experimental hypercholesterolemia. Single-kidney function and hemodynamic responses to endothelium-dependent challenge were assessed in pigs after 12 weeks of hypercholesterolemia, hypercholesterolemia chronically supplemented with thalidomide (4 mg/kg per day), and normal controls. Renal microvascular architecture was then studied ex vivo using 3D microcomputed tomography imaging and inflammation, angiogenesis, and oxidative stress explored in renal tissue. The density of larger microvessels (200 to 500 μm) was selectively decreased in hypercholesterolemia plus thalidomide and accompanied by a decreased fraction of angiogenic, integrin β3-positive microvessels (9.9%±0.9% versus 25.5%±1.7%; P<0.05 versus hypercholesterolemia), implying decreased angiogenic activity. Furthermore, thalidomide increased renal expression of endothelial NO synthase and decreased tumor necrosis factor-α and renal inflammation but did not decrease oxidative stress. Thalidomide also decreased basal renal blood flow and glomerular filtration rate but normalized the blunted renal hemodynamic responses in hypercholesterolemia. Attenuated inflammation and pathological angiogenesis achieved in hypercholesterolemia by thalidomide are accompanied by restoration of renovascular endothelial function but decreased basal renal hemodynamics. This study, therefore, suggests that neovascularization in the hypercholesterolemic kidney is a compensatory mechanism that sustains basal renal vascular function. (Hypertension. 2007;50:729-736.)

Key Words: kidney ■ thalidomide ■ hypercholesterolemia ■ microcirculation ■ inflammation

Hypercholesterolemia (HC) is present in >50% of the middle-aged adult US population and constitutes a risk factor for atherosclerosis. Notably, before the presence of overt plaques, lipid abnormalities can impair both the function and structure of large and small vessels of many types of vascular beds and can damage target organs.

Lipid abnormalities are frequently associated with renal disease and dysfunction and may trigger renal injury at an early stage. HC may increase renal oxidative stress, which may impair the function of the microvascular network (MV) by decreasing NO bioavailability and favoring vasoconstriction, endothelial dysfunction, inflammation, fibrosis, and tissue injury. Inflammation is involved in many forms of renal injury but may also trigger compensatory mechanisms to offset tissue injury. For example, increased oxidative stress and, to a lesser degree, inflammation, are considered the main mechanisms leading to vascular endothelial dysfunction. Furthermore, we have shown previously that diet-induced HC also results in renal MV proliferation, possibly because of renal inflammation. Proliferation of pathological and dysmorphic MV, showing decreased intercellular contact and increased permeability, may culminate in renal tissue injury and interstitial fibrosis. On the other hand, increased MV density can contribute to tissue perfusion but is not fully effective in HC because it was insufficient to preserve the endothelial function of the kidney. Therefore, the role of HC-induced inflammation and neovascularization in the kidney remained unclear.

Thalidomide is a racemic glutamic acid derivative with prominent antiangiogenic properties approved in the United States for erythema nodosum leprosum and multiple myeloma. In addition, its use in various inflammatory and oncologic conditions is under intense investigation. Indeed, growing evidence shows the anti-inflammatory effects of this drug achieved through downregulation of key cytokines, such as nuclear factorκB, tumor necrosis factor (TNF)-α, and...
These factors are all highly expressed in the HC kidney and promote renal inflammation and MV endothelial dysfunction.\textsuperscript{17,18} The antiangiogenic and anti-inflammatory properties of thalidomide, therefore, provide the opportunity to study the role of renal neovascularization induced by inflammation in HC. Hence, the present study was designed to test the hypothesis that chronic thalidomide supplementation would attenuate intrarenal inflammation, decrease MV proliferation, and consequently impair the basal hemodynamics and function of the HC kidney.

Methods

The Institutional Animal Care and Use Committee approved all of the procedures. Twenty domestic pigs (50 to 60 kg) were studied after 12 weeks of normal control (n=7), a 2% HC (n=7)\textsuperscript{6,18–20} diet, and HC with oral supplementation with thalidomide at a standard dose (HC+thal) 4 mg/kg per day, Celgene Co\textsuperscript{12}; n=6 per group).

On the day of the in vivo studies, animals were anesthetized with a mixture of ketamine (0.2 mg/kg per minute) and xylazine (0.03 mg/kg per minute) in normal saline. Under sterile conditions and fluoroscopic guidance, via a neck cutdown, a pigtail catheter was placed in the superior vena cava and an 8F arterial catheter in the abdominal aorta above the renal arteries. Blood pressure was measured using the side arm of a carotid arterial sheath. In vivo electron-beam computed tomography (CT) flow studies were then performed to assess basal and challenged renal blood flow (RBF) and glomerular filtration rate (GFR). We have shown previously that, using electron-beam CT, we can obtain accurate, repeated, and noninvasive quantifications of RBF and GFR\textsuperscript{5,6,11,19–24} in the intact single kidney in vivo, allowing evaluation of renal and renovascular function. Briefly, a sequential acquisition of 40 consecutive scans followed a central venous injection of the contrast medium iopamidol (0.5 cc/kg per 2 seconds). These were repeated during suprarenal aortic infusion of acetycholine (Ach, 5 mg/kg per minute) to test intrarenal MV endothelium-dependent responses. Blood samples were collected from the inferior vena cava for measurement of lipid profile (Roche Laboratories),\textsuperscript{6} oxidized low-density lipoprotein (LDL; Mercodia),\textsuperscript{24} and circulating TNF-\(\alpha\) (R&D Systems).\textsuperscript{25}

The pigs were killed 3 days after completion of the in vivo studies (to allow elimination of contrast media and/or vasoactive agents) with a lethal intravenous dose of sodium pentobarbital (100 mg/kg, Sleepaway, Ft Dodge Laboratories, Inc.). Kidneys were removed using a retroperitoneal incision and were immersed in Kreb’s solution containing heparin (10 U/mL) to prevent drying. A lobe of tissue was immersed in 10% buffered formalin (Sigma), and a segmental artery perfusing the intact end of the kidney was cannulated and prepared for micro-CT. This imaging technique permits assessment of the 3D pattern of MV structure in situ, providing powerful means for studying their spatial distribution and connectivity. We have shown the feasibility of studying renal architecture with micro-CT in experimental HC\textsuperscript{6,11} and renovascular disease.\textsuperscript{24,28} Another lobe of renal tissue was removed from 1 end of the kidney, shock-frozen in liquid nitrogen, and stored at \(-80^\circ\mathrm{C}\).\textsuperscript{5,7,22–24}

In vitro studies were then performed to assess the expression of proinflammatory, angiogenic, and fibrogenic factors in the kidney. Renal redox status was evaluated by assessing the in situ production of superoxide anion, detected by fluorescence microscopy using dihydroethidium,\textsuperscript{26} by the expression by Western blotting of the radical forming enzymes reduced nicotinamide-adenine dinucleotide phosphate-oxidase (p47phox and p67phox), xanthine oxidase, and uncoupled endothelial NO synthase (eNOS), the main sources of reactive oxygen species (ROS). Furthermore, the endogenous anti-oxidant activity was investigated by assessing the expression of Cu/Zn superoxide dismutase (SOD), the predominant form of SOD in blood vessels,\textsuperscript{29} and the ratio of reduced/oxidized glutathione (Oxis Inc), as described previously.\textsuperscript{26,27} Western blotting and PCR were also used to assess renal protein and/or mRNA expression of TNF-\(\alpha\), vascular endothelial growth factor (VEGF), plasminogen (Plg), and pre-pro endothelin (ET)-1 and its specific ET-A and ET-B receptors. Renal expression of Plg was used as a marker of vascular permeability\textsuperscript{31} that characterizes angiogenic MV. In addition, MV density and proliferation were assessed by micro-CT (see below) and by renal immunoreactivity for integrin \(\beta_3\). Renal inflammation and angiogenic vessels were investigated in representative 5-\(\mu\)m-thick renal midhilary cross-sections (1 per animal) stained with CD-3 and integrin \(\beta_3\), respectively.

Micro-CT

The kidney was perfused with a radio-opaque silicone polymer (Microfil MV122, Flow Tech, Inc) and scanned at 0.5\textsuperscript{15} increments using a micro-CT scanner, as described previously.\textsuperscript{8,24,28} For details, see the online supplement, available at http://hyper.ahajournals.org.

Real-Time Quantitative PCR

To investigate the renal mRNA expression of eNOS, TNF-\(\alpha\), pre-pro ET-1, ET-A, and ET-B, real-time PCR (DNA engine OPTICON, MJ Research) was performed using a SYBR Green JumpStart Taq ReadyMix kit (Sigma). Either human or porcine (when available) gene-specific sequences were used, as described previously.\textsuperscript{8,5,24} For details, see the online supplement.

Data Analysis

Electron-Beam CT Analysis

Manually traced regions of interest were selected in electron-beam CT images in the aorta, renal cortex, and medulla, and their densities were sampled over time. Time-density curves were generated and used to calculate single-kidney RBF and GFR using previously validated methods.\textsuperscript{5,24} For details, see the online supplement.

Micro-CT Analysis

3D volume images were reconstructed and analyzed with the Analyze software package (Biomedical Imaging Resource, Mayo Clinic) as described previously.\textsuperscript{8,24,28} For details, see the online supplement.

Renal Histology

Midhilar cross-sections of the kidney (1 per animal) were examined using a computer-aided image-analysis program (MetaMorph, Meta Imaging Series 6.3.2).\textsuperscript{5,7,19-24,25} For quantification of vessels expressing integrin \(\beta_3\), all of the vessels were manually counted in each slide (1 per animal), and the fraction of integrin plus vessels per slide was calculated. For additional details, see the online supplement.

Statistical Analysis

Results are expressed as mean±SEM. Comparisons within groups were performed using paired Student’s t test and among groups using ANOVA, with the Bonferroni correction for multiple comparisons, followed by unpaired Student’s t test. Statistical significance was accepted for \(P<0.05\).

Results

Body weight and mean arterial pressure were similar among the groups, whereas total and LDL cholesterol, as well as circulating oxidized LDL levels, were similarly elevated in
HC and HC+thal animals compared with normal animals (Table).

**MV 3D Architecture and Angiogenic Factors**

The density of larger MV (200 to 500 μm) was similar in normal and HC but selectively decreased in HC+thal animals (Figure 1). This was accompanied by decreased renal expression of VEGF and Plg, as well as a reduction in the fraction of integrin β3-positive vessels compared with untreated HC (Figure 1), implying a reduction in renal angiogenic activity. As we have shown before, the HC kidney showed a significant increase in the density of smaller MV (<200 μm)

### Table. Systemic Characteristics and Basal Single-Kidney Hemodynamics (Mean±SEM) in Normal (n=7), HC (n=7), and HC+Thal Pigs (n=6 per Group)

<table>
<thead>
<tr>
<th>Variables</th>
<th>Normal</th>
<th>HC</th>
<th>HC+Thal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, kg</td>
<td>56.8±2.1</td>
<td>58.9±1.0</td>
<td>55.3±1.8</td>
</tr>
<tr>
<td>Mean arterial pressure, mm Hg</td>
<td>99.6±5.2</td>
<td>101.4±5.4</td>
<td>102.1±4.6</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>1.9±0.2</td>
<td>10.3±0.8*</td>
<td>9.4±1.1*</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>0.8±0.06</td>
<td>7.3±0.9*</td>
<td>6.8±1.5*</td>
</tr>
<tr>
<td>Oxidized LDL, units/L</td>
<td>9.6±1.0</td>
<td>20.4±2.1*</td>
<td>20.5±1.9*</td>
</tr>
<tr>
<td>Renal blood flow, mL/min</td>
<td>542.9±26.1</td>
<td>521.6±43.6</td>
<td>433.9±33.9†</td>
</tr>
<tr>
<td>Glomerular filtration rate, mL/min</td>
<td>71.1±2.3</td>
<td>64.3±4.0</td>
<td>58.4±2.7*</td>
</tr>
</tbody>
</table>

*P<0.05 vs normal; †P<0.05 vs HC.

Figure 1. Top left, Representative 3D microtomographic images (displayed at 40-μm voxel size) showing interlobar (I), arcuate (A), radial (R), and smaller branching orders (S) cortical MVs from normal, HC, and HC+thal pigs. Bottom, Quantification of cortical MV density, average diameter, and vascular volume fraction. Right, Representative immunoblots and densitometric quantification demonstrating renal protein expression of VEGF (top), quantification of renal microvascular immunoreactivity to integrin β3 (middle), and renal protein expression of Plg (bottom). Despite a similar increase in the transmural MV density in HC and HC+thal, treated animals showed decreased density of large MV (200 to 500 μm) and, consequently, decreased renal vascular volume fraction but a reciprocal increased density of small (<200 μm) MV compared with both normal and HC animals. The reduction in renal expression of VEGF and integrin β3-positive vessels by thalidomide implies a decrease in angiogenic activity and suggests that the selective increase in density of small MV resulted from recruitment of pre-existing vessels. Furthermore, HC+thal showed decreased expression of Plg, implying a decrease in vascular leakage. *P<0.05 vs normal. †P<0.05 vs HC.
compared with normal, which was interestingly accentuated in HC+thal (Figure 1). The reciprocal increase in the small MV density resulted in the transmural spatial MV density that was overall similarly increased in HC and HC+thal compared with normal (81.0±12.0, 110.8±20.1, and 48.5±11.9 MV/cm², respectively; P<0.05 versus normal). However, because of the increased density of small vessels relative to large MV, the average diameter and vascular volume fraction of cortical MV were significantly reduced in HC+thal compared with normal kidneys (Figure 1).

Renal Inflammation
Circulating levels and renal expression of proinflammatory TNF-α and renal CD3⁺ cells were increased in HC but normalized in thalidomide-treated animals, indicating attenuation in renal inflammation (Figure 2).

MV and Renal Function
Basal RBF and GFR in HC were not different from normal but were reduced in HC+thal pigs (Table). Infusion of Ach was not associated with a persistent change in blood pressure, as we have shown previously.⁵ The increase in RBF in response to Ach was blunted in HC (+6.4±8.4%; P value not significant versus baseline) but was significant in both normal and thalidomide-treated HC animals (+31.6±8.6% and +33.8±8.9%, respectively; P<0.05 versus baseline for both), whereas GFR responses to Ach were not different among the normal, HC, and HC+thal groups (+32.4±8.4%, +35.4±12.1%, and +43.5±8.9%, respectively; P<0.05 versus baseline for all; Figure 3). Notably, the blunted renal expression of eNOS in HC and the increased expression of the ET-A receptor were normalized in HC+thal kidneys (Figure 3), suggesting a vasodilator permissive milieu. However, the expressions of uncoupled eNOS, xanthine oxidase, and reduced nicotinamide-adenine dinucleotide phosphate-oxidase were increased in HC kidneys, and most were further elevated in HC+thal, suggesting the potential for increasing renal oxidative stress (Figure 4). In contrast, renal reduced/oxidized glutathione and CuZn SOD expression were also significantly increased in HC+thal compared with both normal and HC, implying augmented potential for ROS scavenging as well. Consequently, the overall abundance of ROS in the tissue (as reflected in dihydroethidium fluorescence) and index of oxidative stress were similarly elevated in HC and HC+thal compared with normal animals.

Discussion
This study shows that a 12-week regimen of thalidomide supplementation significantly decreased renal inflammation and arrested MV proliferation in the HC kidney. These effects were accompanied by decreased RBF and GFR, suggesting that renal neovascularization in HC contributed to maintaining basal renal hemodynamics and vascular function. Nevertheless, despite lingering renal oxidative stress, thalidomide also improved endothelium-dependent MV function in HC, underscoring the contribution of inflammation to renal dysfunction.

The role of lipid abnormalities as a prominent risk factor for renal disease progression has been increasingly recognized,³³ and deterioration of renal function is conversely also associated with and promotes alterations in lipid metabolism. We have shown previously that diet-induced HC led to a
significant increase in oxidative stress and renal inflammation associated with renovascular endothelial dysfunction and MV proliferation and remodeling.\textsuperscript{5,6,18} Inflammation is a complex process involving changes in hemodynamics, vascular reactivity, and activation and migration of inflammatory cells.\textsuperscript{34} One of the defense mechanisms by which inflammation facilitates healing is formation of new vessels to sustain perfusion of ischemic or diseased tissues.\textsuperscript{35} Indeed, we have shown previously that the HC kidney exhibits neovascularization, possibly reflecting a compensatory mechanism to episodes of inadequate perfusion, although renovascular endothelial function remained attenuated in HC. Hence, the role of the neovessels in the HC kidney remained unclear.

Thalidomide is an immunomodulatory agent known not only for its anti-inflammatory actions but also for its capability for arresting angiogenesis.\textsuperscript{13} The mechanism of antiangiogenic activity of thalidomide may include decreasing cell migration\textsuperscript{36} or downregulation of proangiogenic factors such as VEGF.\textsuperscript{37} Accordingly, in the current study, the HC+thal kidney showed decreased expression of VEGF accompanied by a decrease in the density of large renal MV (between 200 and 500 $\mu$m in size) compared with both normal and HC animals. Intriguingly, however, whereas HC showed increased density of small MV (<200 $\mu$m) compared with controls, HC+thal animals showed a higher density of small MV. The smaller diameter, lower Plg expression (implying decreased vascular leakage\textsuperscript{31}), and decreased abundance of integrin $\beta_3$-positive vessels in HC+thal supports the notion that the increased density of small MV in these animals was not because of angiogenic microvessels,\textsuperscript{38} which are characterized by increased permeability and larger diameter.\textsuperscript{39} Furthermore, MV endothelial function in vivo was importantly normalized in HC+thal kidneys. Hence, these smaller MVs in HC+thal might reflect recruitment of pre-existing MV to compensate for the regression of some of the upstream vasculature. This reciprocal increase in MV <200 $\mu$m resulted in an overall MV density that was similarly elevated in HC and HC+thal compared with normal animals. On the other hand, the selective decrease in larger MV by thalidomide may account for the reduced vascular volume fraction and thereby RBF and GFR, suggesting that those new vessels served to sustain basal renal perfusion in HC. Nevertheless, the blunted hemodynamic responses to endothelium-dependent challenge and increased MV permeability in HC (implying renovascular endothelial dysfunction) suggested that those angiogenic MVs in the HC kidney were dysfunctional. The unimpaired GFR response to Ach in HC underscores its regulation that may be different than that of vascular resistance.

The current study also shows that experimental HC upregulates both the systemic and renal levels of the proinflammatory mediator TNF-$\alpha$, which has been implicated in the pathogenesis of many inflammatory diseases of the kidney. TNF-$\alpha$ is synthesized by resident glomerular cells in response to...
to injury and by infiltrating macrophages and can promote renal dysfunction via direct cytotoxicity, vasoconstriction, and decreased RBF and by the recruitment of neutrophils and monocytes. Elevated TNF-α can also decrease GFR by promoting glomerular fibrin deposition, cellular infiltration, and vasoconstriction and may induce the synthesis of other proinflammatory mediators. Importantly, thalidomide treatment significantly reduced both the circulating and renal protein and mRNA expressions of TNF-α in HC and decreased infiltration of CD3+ cells, suggesting attenuated renal inflammation.

Increased abundance of superoxide anion is considered to be one of the main mechanisms mediating renal endothelial dysfunction. Nevertheless, inflammatory states may be associated with abnormal vascular function, likely as a result of downregulated eNOS and consequently decreased synthesis and availability of NO. The current study shows that the blunted renovascular endothelial function is accompanied by a decrease in both renal protein and mRNA expression of TNF-α in HC and decreased infiltration of CD3+ cells, suggesting attenuated renal inflammation.

Figure 4. Top left, Representative renal protein expression and quantification of uncoupled eNOS, xanthine oxidase (XO), reduced nicotinamide-adenine dinucleotide phosphate-oxidase p47phox and p67phox subunits, and CuZn superoxide dismutase (CuZn SOD). Bottom left, ratio of reduced/oxidized glutathione (GSH/GSSG). Right, Renal production of superoxide anion disclosed by dihydroethidium (DHE) fluorescence microscopy (×40) in normal, HC, and HC+thal animals. Thalidomide showed a renal pro-oxidant effect accompanied by evidence suggestive of increased scavenging as well. *P<0.05 vs N; †P<0.05 vs HC; #P<0.08 vs N.

In contrast to the improved endothelial function and decreased inflammation, the effects of thalidomide on oxidative stress could potentially be deleterious. Renal expression of both reduced nicotinamide-adenine dinucleotide phosphate oxidase and uncoupled-eNOS, which are known sources of ROS, was significantly increased in HC+thal compared not only with normal, but with HC animals as well, although renal expression of xanthine oxidase was similarly increased.

ROS have a distinct constrictor effect on the afferent arteriole, which may also account for the lower RBF and GFR observed in HC+thal animals. However, thalidomide also augmented in the HC kidney the ratio of renal reduced/
oxidized glutathione and the expression of CuZn SOD. Hence, in parallel with its pro-oxidant effects, thalidomide promoted renal ROS scavenging, which may explain why superoxide abundance in situ was not higher than in HC. Yet, the remaining ROS and oxidized LDL might have contributed to the decrease in basal RBF and GFR, because thalidomide has been suggested to exacerbate pre-existing renal impairment⁴⁷ and might have potentially aggravated their vasoconstrictor effects.

**Perspectives**

The current study shows for the first time that chronic thalidomide treatment decreased renal inflammation and angiogenesis in HC, thereby restoring renovascular endothelial function but at the same time decreasing basal RBF and GFR. Interestingly, the early stages of renal inflammation and oxidative stress are promoted in HC without a concomitant increase in arterial pressure, possibly because of impaired tubular concentration mechanisms.⁴⁸ Nonetheless, inflammation and oxidative stress could still eventually promote hypertension in HC and atherosclerotic patients and, thus, potentially thalidomide might indirectly blunt development of hypertension. In conclusion, these results demonstrate the dual role of inflammation as both a pathological process and defense mechanism during the evolution of renal injury in early atherosclerosis.

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**Disclosures**

None.

**References**


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