Endothelin-A Receptor Blockade Improves Renal Microvascular Architecture and Function in Experimental Hypercholesterolemia

Alejandro R. Chade,* James D. Krier,* Stephen C. Textor,* Amir Lerman,† and Lilach O. Lerman*†

Department of Internal Medicine, Divisions of *Nephrology and Hypertension and †Cardiovascular Diseases, Mayo Clinic College of Medicine, Rochester, Minnesota

Hypercholesterolemia (HC) may trigger early renal injury, partly by impairing the function or the structure of renal microvessels (MV). The endothelin (ET) system is upregulated in HC and can have an impact on the renal microcirculation by regulating MV tone, growth factors, and remodeling. It was hypothesized that ET-A blockade would protect the HC kidney by improving the function and attenuating the damage of intrarenal MV. Single-kidney function and hemodynamic responses to endothelium-dependent challenge were assessed in pigs after 12 wk of experimental HC, HC and chronic supplementation with the ET receptor A blocker ABT-627 (HC/ET-A, 0.75 mg/kg per d), and normal controls. Renal MV architecture then was studied ex vivo using three-dimensional microcomputed tomography imaging, and growth factors and remodeling pathways were explored in renal tissue. The HC kidney showed increased MV density compared with normal (77.68 ± 5.1 versus 62.9 ± 4.8 vessels/cm²; P = 0.04) but blunted endothelial function. Chronic ET-A blockade in HC upregulated renal vascular growth factors, further increased renal MV density (139.9 ± 8.4 vessels/cm²; P = 0.001 versus normal and HC), and decreased renal tissue and MV remodeling. Furthermore, ET-A blockade in HC decreased MV tortuosity and improved MV endothelial function, suggesting accelerated stabilization and maturation of neo-vessels. Modulation of renal MV architecture and function in HC is mediated partly by the endogenous ET system. Notably, ET-A blockade enhanced the proliferation and facilitated the maturation of renal MV in the HC kidney and improved renal MV remodeling and function. This study suggests novel renoprotective effects of ET-A blockers and supports further exploration of strategies that target the ET pathway in HC and atherosclerosis.


Atherosclerosis, one of the major causes of premature death in the United States today (1,2), is a generalized and inflammatory vascular disease that frequently is associated with renal disease (3) and dysfunction (4). Hypercholesterolemia (HC), a risk factor for atherosclerosis, is present in approximately 50% of the middle-aged adult US population (1). Dyslipidemia can alter vasoactive regulation in both large and small vessels and impair both the function and the structure of many types of vascular beds. Increased systemic and local (5) activation of the endothelin (ET) system, which may be mediated partly by oxidized LDL (ox-LDL) (6), has been observed consistently in both HC (7,8) and atherosclerosis (9,10). ET-1 is a potent and long-lasting renal vasoconstrictor that is involved in the regulation of vascular tone (5), has pronounced athrogenic and mitogenic properties, and contributes to the progression of atherosclerosis.

In the kidney, lipid abnormalities may trigger renal injury at an early stage and often accompany and aggravate renal disease. We previously showed that diet-induced HC upregulates both renal ET-1 and its ET-A receptor (11,12) and that through the ET-A receptor, ET-1 mediates renal endothelial dysfunction and increases oxidative stress, inflammation, and fibrogenic activity in the HC kidney (13). We also showed that HC induced growth factor expression and microvascular (MV) proliferation in the kidney, possibly in an attempt to preserve basal renal perfusion in a milieu that is rich in vasoconstrictors and in the face of MV remodeling. However, renovascular endothelial function in HC remained attenuated.

Importantly, ET can damage the microcirculation by regulating vascular growth–promoting factors and inducing MV remodeling (14–18). MV remodeling and dysfunction are important mechanisms of organ damage (19) that influence the progression and may interfere with therapeutic interventions for vascular diseases (20). We previously showed that the HC kidney has a significant increased MV proliferation, associated with mild MV fibrosis and wall thickening, indicators of early MV damage (13,21). Activation of the endogenous ET system may well play a role in this deleterious process in the HC kidney but remains to be elucidated.

Microcomputed tomography (micro-CT) imaging permits assessment of the three-dimensional (3-D) pattern of MV struc-
ture in situ, providing powerful means for the study of spatial distribution and connectivity of MV in the kidney as an organ. We have demonstrated the feasibility of studying renal architecture with micro-CT in experimental HC (21,22) and renovascular disease (23). In addition, we have applied electron-beam CT (EBCT) to obtain accurate and noninvasive quantifications of single-kidney volume, regional perfusion, renal blood flow (RBF), and GFR (24–30) of the intact kidney in vivo. Therefore, in this study, these powerful imaging techniques were used to test the hypothesis that ET-A blockade would attenuate intrarenal MV damage in HC and consequently preserve the hemodynamic and function of the HC kidney.

Materials and Methods

The Institutional Animal Care and Use Committee approved all of the procedures. Twenty domestic pigs (50 to 60 kg) were studied after 12 wk of normal (n = 7), 2% HC (n = 7) (25,31), or HC diet orally supplemented with the selective ET-A blocker ABT-627 (HC+ET-A; n = 6) on a weight-adjusted scale to maintain a dosage of 0.75 mg/kg per d (32). ABT-627 is an orally active, nonpeptide selective ET-A receptor antagonist that has been characterized fully and has a binding Ki for the ET-A receptors approximately 2000-fold greater than for the ET-B receptors (0.035 and 69.5 nmol/L, respectively) (33,34).

On the day of the in vivo studies, each pig was anesthetized with 0.5 g of intramuscular telazol (5 mg/kg) and xylazine (2 mg/kg), intubated, and mechanically ventilated with room air. Anesthesia was maintained with intravenous ketamine (0.2 mg/kg per min) and xylazine (0.03 mg/kg per min) in normal saline. Under sterile conditions and fluoroscopic guidance, a pigtail catheter was placed in the superior vena cava and an 8-F arterial catheter was placed in the abdominal aorta above the renal arteries. In vivo EBCT flow studies then were performed, as previously detailed (24–28), for assessment of basal regional-renal perfusion, RBF, GFR, and tubular function. Briefly, this involved sequential acquisition of 40 consecutive scans after a central venous injection of the contrast medium iopamidol (0.5 ml/kg per 2 s), which were fused, RBF, GFR, and tubular function. Briefly, this involved sequential acquisition of the selective ET-A blocker ABT-627 (HC+ET-A; n = 6) on a weight-adjusted scale to maintain a dosage of 0.75 mg/kg per d (32). ABT-627 is an orally active, nonpeptide selective ET-A receptor antagonist that has been characterized fully and has a binding Ki for the ET-A receptors approximately 2000-fold greater than for the ET-B receptors (0.035 and 69.5 nmol/L, respectively) (33,34).

Ki for the ET-A receptors approximately 2000-fold greater than for the ET-B receptors (0.035 and 69.5 nmol/L, respectively) (33,34).

Real-Time Quantitative PCR

For investigation of the expression of VEGF, MMP-2, and TIMP-2 mRNA, real-time PCR (DNA engine OPTICON; MJ Research, Waltham, MA) was performed using SYBR Green JumpStart TaqReadyMix kit (Sigma). Briefly, 12.5 μl of SYBR Green JumpStart TaqReadyMix, 0.25 μl of internal reference, 0.5 μl of primer 5’, 0.5 μl of primer 3’, 1 μl of cDNA, and 10.25 μl of DEPC water reached a 25-μl final reaction volume. Either human or porcine (when available) gene specific sequences were used as described previously (12,35). The relative amount of mRNA, normalized to an internal control glyceraldehyde-3-phosphate dehydrogenase and relative to a calibrator (normal), was calculated by 2−ΔΔCt. Real-time quantitative PCR results were quantified and expressed as percentage change in copy numbers compared with the normal group.

Western Blotting

Standard blotting protocols were followed, as described previously (26), using specific polyclonal antibodies against VEGF, TSP 1/2, and the AT-1 receptors (Santa Cruz Biotechnology, Santa Cruz, CA; 1:200 for all), TGF and angiotropin-1 (Novus Biologicals, Littleton, CO; 1:500), and aAb against both the precursor and active MMP-2 (Chemicon International, Temecula, CA; 1:200). β-Actin (Sigma; 1:500) were used as loading controls. Intensities of the protein bands (one per animal) were determined using densitometry, quantified, and averaged.

Data Analyses

EBCT Analysis. Manually traced regions of interest were selected in EBCT images in the aorta, renal cortex, and medulla, and their densities were sampled. Time-density curves were generated and fitted with extended y-variate curve-fits, and the area enclosed under each segment of the curve and its first moment were calculated using the curve-fitting parameters (29). These were used to calculate renal regional perfusion (ml/min per g tissue), single-kidney GFR, and RBF, using previously validated methods (24–30,36).

Micro-CT Analysis. 3-D volume images were reconstructed and analyzed with the Analyze software package (Biomedical Imaging Resource; Mayo Clinic, Rochester, MN). The renal cortex was tomo-
The spatial density and average diameter of cortical MV was greater in HC compared with normal kidneys (77.6 ± 5.1 versus 62.9 ± 4.8 vessels/cm²; *P* = 0.04), most evidently in the small vessels (<200 μm; Figure 3) of the inner cortex. Notably, HC+ET-A kidneys showed MV with smaller diameter and further increased spatial density (139.9 ± 8.4 vessels/cm²; *P* = 0.001 versus normal and HC) in all of the cortical regions, although average vascular volume fraction was similar among the groups (Figure 3). Conversely, HC+ET-A kidneys showed a significant decrease in MV tortuosity (an index of angiogenic vessels) compared with both normal and HC. Furthermore, in HC, MV density showed a moderate but significant inverse correlation with the response to Ach of both RBF (*r* = 0.520) and GFR (*r* = 0.65), suggesting an association between increased MV density and renal endothelial dysfunction, whereas in HC+ET-A, no such correlation was observed. In addition, renal expression of the AT-1 receptor was similarly increased in both HC and HC+ET-A and does not support the contention that the effects of ET-A blockade were mediated by downregulation of the tissue renin-angiotensin system. However, whereas total Akt was similar among the groups, HC pigs showed a decrease in p-Akt that was normalized in HC+ET-A, suggesting improved maturation of MV (37). Consequently, renal mRNA and protein expression of VEGF, which was slightly increased in HC (but did not reach statistical significance), was significantly elevated further in HC+ET-A. Finally, renal expression of TSP 1/2 was higher in the HC kidney but normalized in HC+ET-A, whereas angiopoietin-1 was similar in both normal and HC but

### Table 1. Systemic characteristics and basal single-kidney hemodynamics (mean ± SEM) in normal, HC, and HC+ET-A

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Normal (n = 7)</th>
<th>HC (n = 7)</th>
<th>HC+ET-A (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>1.7 ± 0.1</td>
<td>10.5 ± 0.9b</td>
<td>8.8 ± 1.9b</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>0.7 ± 0.08</td>
<td>7.6 ± 0.8b</td>
<td>6.9 ± 1.9b</td>
</tr>
<tr>
<td>Oxidized LDL (mmol/L)</td>
<td>9.9 ± 0.8</td>
<td>20.8 ± 2.6b</td>
<td>16.9 ± 0.8bc</td>
</tr>
<tr>
<td>Mean arterial pressure (mmHg)</td>
<td>100.9 ± 3.7</td>
<td>102.7 ± 4.5</td>
<td>103.1 ± 6.1</td>
</tr>
<tr>
<td>Plasma renin activity (ng/ml)</td>
<td>0.49 ± 0.07</td>
<td>0.47 ± 0.1</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>Renal volume (ml)</td>
<td>144.7 ± 7.6</td>
<td>128.1 ± 8.1</td>
<td>131.8 ± 10.7</td>
</tr>
<tr>
<td>Renal blood flow (ml/min)</td>
<td>518.6 ± 31.7</td>
<td>514.5 ± 54.4</td>
<td>496.6 ± 50.1</td>
</tr>
<tr>
<td>GFR (ml/min)</td>
<td>75.1 ± 4.6</td>
<td>69.2 ± 5.0</td>
<td>76.9 ± 6.0</td>
</tr>
<tr>
<td>Perfusion (ml/min per ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cortex</td>
<td>4.5 ± 0.3</td>
<td>4.4 ± 0.4</td>
<td>4.2 ± 0.2</td>
</tr>
<tr>
<td>medulla</td>
<td>2.5 ± 0.4</td>
<td>3.1 ± 0.6</td>
<td>3.1 ± 0.4</td>
</tr>
</tbody>
</table>

\(^{a}\)ET-A; endothelin receptor blocker; HC, hypercholesterolemic.  
\(^{b}\)P < 0.05 versus normal.  
\(^{c}\)P < 0.05 versus HC.
elevated in HC+ET-A (Figure 4), again supporting MV maturation.

**Renal MV Morphology and Remodeling**

Glomerulosclerosis was not observed in any of the groups, whereas tubulointerstitial fibrosis was evident in HC but normalized after ET-A blockade (Figure 5A). Renal sections that were stained with trichrome showed increased MV media-to-lumen ratio and perivascular fibrosis in the HC kidney (Figure 5A), which were accompanied by increased renal expression of tTG (Figure 5A), suggesting increased MV remodeling. All of these changes were substantially attenuated in HC+ET-A. Conversely, the protein (but not the mRNA) expression of both the pro- and active forms of MMP-2 were blunted in HC, whereas TIMP-2 was increased, as we previously showed (12) (Figure 5B). Notably, ET-A blockade normalized the expression of tTG and augmented both the protein and mRNA expression of MMP-2 and decreased TIMP-2 in HC.

**Discussion**

This study demonstrates the renoprotective effect of chronic ET-A receptor blockade in experimental HC. ET-A blockade in HC increased renal MV density and improved their function,
likely as a result of enhanced proliferation and maturation of new vessels. In addition, HC + ET-A kidneys showed significant attenuation of renal MV and tissue remodeling. Our study suggests novel effects of ET-A blockade on intrarenal MV and a potential role for these agents for protecting the kidney in HC and atherogenesis.

The prominent role of lipid abnormalities for renal disease progression have been recognized increasingly (38). We previously showed that even a short-term exposure to high cholesterol blunts renovascular endothelial function by favoring vasoconstriction and decreasing the buffering effects of endogenous vasodilators, such as nitric oxide (NO), and elicits tissue injury, an effect that is mediated mainly by increased oxidative stress (11,13,25). The increase in both oxidative stress (39) and inflammation (40,41) in the HC kidney likely activates local cytokines and growth factors and, thereby, fibrosis. Possibly, the persistent oxidative stress and inflammation in HC may underlie renal MV endothelial dysfunction and remodeling that can decrease renal perfusion further. The pro-oxidant state also is responsible for the decreased matrix degradation (12,42) in the HC kidney, a process that normally limits renal damage and preserves its structure. Furthermore, we also showed increased formation of intrarenal neo-vessels in experimental HC (21,22) and that neovascularization was accompanied by upregulation of VEGF, an effect that likely is mediated by increased endogenous oxidative stress and inflammation. Because the number of glomeruli does not increase after birth, in the kidney, these new vessels likely do not contribute to glomerular filtration but rather to support tissue perfusion. Although the HC-induced MV proliferation could be a compensatory response to sustain renal perfusion, the newly developed vessels in the HC kidney often are not fully functional. Indeed, their failure to restore renal endothelial function (21,25) suggested that the new vessels were insufficient in number, damaged, or dysfunctional.

Neovascularization involves a sequence of events such as cell proliferation, migration, and differentiation of endothelial cells; remodeling of extracellular matrix (ECM); and functional maturation of the newly assembled vessels, all of which are crucial for developing neo-vessels. New vessels may be needed to
facilitate adequate oxygen delivery to ischemic tissues but also may be part of a pathophysiologic process, such as cancer (43), inflammation (40), or exposure to cardiovascular risk factors (44). It is interesting that our study shows that ET-A blockade in the HC kidney resulted in a significant expansion of the MV bed. The increased MV density in HC + ET-A was associated with a significant increase in the expression of VEGF, a positive regulator of both physiologic and pathophysiologic neovascularization. However, overexpression of VEGF alone may induce neo-vessels that are leaky, abnormally large in diameter (45), and therefore not fully functional. Notably, ET-A blockade normalized renal Akt activation, a key mediator for VEGF transcription, which also has a role in improving the maturation and permeability of angiogenic vessels (37). Similarly, renal expression of angiopoietin-1 was elevated in the HC + ET-A kidney. Angiopoietin-1 stabilizes and accelerates maturation and integrity of new vessels by reducing the endothelial gaps and, therefore, the permeability and plasma leakage, a marker of vascular inflammation (46). In addition, ET-A blockade significantly downregulated renal expression of TSP, an inhibitor of angiogenesis that acts by both binding directly to VEGF and displacing it from endothelial cells (47). Therefore, blockade of the ET-A receptor likely resulted not only in increased MV density but also in stabilization of the new vessels.

In addition, the enhanced MV function in HC + ET-A may have been the result of the improvement in MV remodeling, as reflected in decreased tortuosity and media-to-lumen ratio. We previously showed that HC induced renal tissue and MV damage, partly mediated by upregulation of ET-1 and its ET-A receptor (12,13). Our study extends these observations and shows that HC increased renal expression of tTG, a cross-linking enzyme that modulates MV inward remodeling by its interaction with integrins in the organization of matrix components and vascular remodeling, mainly in situations of sustained vasoconstriction (48). Furthermore, tTG can lead to ECM accumulation (49,50) and thereby may inhibit indirectly the angiogenic response (51). Indeed, the HC kidney showed increased perivascular and tubulointerstitial fibrosis, which possibly limits development of the MV network. The endogenous ET system may mediate an increase in renal TSP (52) and a decrease in renal MMP (53) and thereby favor tTG upregulation (54,55). The MMP, especially MMP-2, play a vital role during angiogenesis by degrading the surrounding ECM and allowing endothelial cell invasion (56). We showed previously (12) that
Despite neovascularization, MMP-2 in fact was downregulated in the HC kidney, which may have contributed to ECM accumulation. It is interesting that chronic ET-A blockade increased MMP-2 and significantly decreased TIMP-2, tTG, and TSP 1/2 in HC, suggesting decreased ECM accumulation and MV remodeling, which likely allowed the expansion of the renal MV bed.

Alternatively, increased spatial density of the MV bed may have resulted partly from a vasodilatory effect of chronic ET-A blockade (57), which dilated and recruited preexisting small MV in the HC kidney, either by decreasing the vasoconstrictor effect that was mediated by the ET-A receptor or by increasing availability of circulating ET to bind to ET-B receptors, which induce renal vasodilation and augment blood flow (58–60). Nonetheless, these recruited or new MV were relatively small, as evidenced by the decrease in average MV diameter in HC/H11001 ET-A, so basal vascular volume fraction and renal perfusion remained unchanged. The increase in number of functional MV throughout the renal cortex may have been responsible for the consequent improvement in overall renovascular endothelial function. In parallel, increased VEGF expression also may have contributed to restoring endothelial function in HC+ET-A kidneys, because VEGF has prominent vasodilatory effects and favors production of NO (61). Therefore, the increased number of intrarenal small MV in HC+ET-A may reflect a combination of both new and preexisting and dilated MV, with decreased vascular wall remodeling and improved endothelial function, which ultimately may have contributed to improve overall renal function.

Importantly, the reduction in oxidative stress after ET-A blockade, disclosed by the attenuated renal superoxide anion and circulating ox-LDL, may have increased further the bioavailability of NO. This in turn may attenuate redox-sensitive inflammatory and fibrogenic cascades that are activated in the HC kidney (13,22), thereby contributing to restoring MV endothelial function and decreasing MV remodeling and renal injury in HC+ET-A. Moreover, we also showed that ET may promote ox-LDL uptake by its specific receptor LOX-1, an effect that is mediated by the ET-A receptor (13). Blockade of ET-A therefore could decrease renal damage, because ox-LDL is cytotoxic to renal mesangial, epithelial, and endothelial cells and may promote cellular necrosis (62).

The beneficial effects of ET-A blockade probably are specific for the disease, tissue, or stage of the disease, because previous studies have shown proangiogenic effects of ET in cancer (63). Of note, its renoprotective effects were achieved without a detectable change in the systemic or renal activity of the renin-angiotensin system (as suggested by the similar PRA and renal expression of the AT-1 receptor), suggesting that the effect of ET blockade probably was not related to modulation of this system. Moreover, additional studies would be needed to evaluate the effect of ET-A blockade during longer exposure to HC.
Our study supports the concept that HC promotes renal injury by altering the renal MV architecture and function, which is mediated partly by the endogenous ET. Although ET-A blockade in HC increased MV density compared with untreated HC, it also improved their maturation and remodeling, contributing to the overall improvement of renal hemodynamics and function. Therefore, our study may suggest a renoprotective effect of chronic ET-A receptor blockade in experimental HC and supports further exploration of strategies that target the ET pathway to preserve the kidney in HC and atherosclerosis.

**Acknowledgments**

This study was supported by grants HL-77131, DK073608, and EB 000305 from the National Institutes of Health. The ET-A blocker ABT-627 was generously provided by Abbott.

We thank Mercedia for development of a kit for measurement of ox-LDL in swine plasma.

**References**


