Simvastatin Decreases Endothelial Progenitor Cell Apoptosis in the Kidney of Hypertensive Hypercholesterolemic Pigs

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Objective—Hypertension and hypercholesterolemia might interfere with renal repair mechanisms. We hypothesized that simvastatin improves the survival of endothelial progenitor cells (EPC) in the renal microenvironment imposed by concurrent renovascular hypertension and dietary hypercholesterolemia (HTC).

Methods and Results—Pigs were studied after 12 weeks of no intervention (n=6), HTC (n=6), or HTC+ oral simvastatin supplementation (80 mg/day, n=5). EPC were also isolated and studied in vitro after exposure to the proapoptotic oxidized low-density lipoprotein with or without coincubation with simvastatin. Renal hemodynamics, function, and endothelial function were evaluated in vivo, and the number of CD34+/KDR+ EPC, apoptosis, oxidative stress, inflammation, and fibrosis in renal tissue studied ex vivo. Compared with normal kidney, the HTC kidney showed endothelial dysfunction and increased oxidative stress, interstitial macrophage filtration, and fibrosis. The number of EPC in the kidney increased, as did their apoptosis (0.85±0.24% versus 0.22±0.07%, P<0.05 versus normal). Simvastatin did not affect blood pressure, cholesterol levels, basal renal function, or number of renal EPC in HTC, but it improved endothelial function; blunted renal oxidative stress, inflammation, and fibrosis; and attenuated EPC apoptosis (to 0.37±0.09%, P<0.05 versus HTC). Simvastatin also significantly decreased oxidized low-density lipoprotein–induced EPC apoptosis in vitro.

Conclusion—EPC are recruited but undergo apoptosis in the HTC kidney, likely because of a hostile microenvironment. Simvastatin rescues renal repair mechanisms in HTC and counteracts renal damage, which may account for its protective effects on the kidney during exposure to cardiovascular risk factors. (Arterioscler Thromb Vasc Biol. 2010;30:00-00-00.)

Key Words: hypertension ■ hypercholesterolemia ■ simvastatin ■ fibrosis ■ apoptosis

Chronic kidney disease (CKD) affects a considerable proportion of the adult population and has become a significant public health problem, because it is also associated with end-stage renal disease, cardiovascular disease, and premature death.1 Cardiovascular risk factors such as dyslipidemia and hypertension (HTN) may lead to deterioration of renal function and have been associated with CKD, progressive atherosclerosis, and cardiac morbidity. Furthermore, clustering of HTN and hypercholesterolemia (HC) further increases the risk for cardiovascular and renal disease.

Cardiovascular risk factors influence renal function and structure by complex neural, humoral, and mechanical mechanisms. An increase in systemic blood pressure results in glomerular capillary wall stretch, endothelial damage, and upregulation of stretch-sensitive enzymes.7 This mechanism and elevated serum cholesterol levels increase oxidative stress and elicit vascular endothelial dysfunction, which may in turn intensify renal cellular injury, apoptosis (programmed cell death), and interstitial fibrosis.8 We have previously shown that early renovascular HTN is associated with increased oxidative stress,9,10 upregulation of inflammatory and profibrotic factors, and impairment of renal hemodynamics, function, and responses to challenge and that the coexistence of HTN with HC (HTC) amplifies renal dysfunction and oxidative stress.11

An important defense mechanism to offset tissue destruction involves recruitment of progenitor and stem cells to the injury site to regenerate and replace damaged cells. Endothelial cells play a key role in renal recovery, because viable endothelium is essential for the survival of mesangial and tubular cells by virtue of its role in oxygen and nutrient delivery.12 Indeed, renal repair may be attributed to both migration and proliferation of resident endothelial cells, as well as recruitment of circulating endothelial progenitor cells (EPC)13 driven by homing signals such as stem cell factor (SCF),14 stromal cell–derived factor-1 (SDF-1),15 and uric acid16 released by the injured tissue. Furthermore, we have demonstrated the important contribution of exogenous autologous EPC to repairing the stenotic kidney in HTN.17 However, a noxious microenvironment characterized by oxidative stress, inflammation, and endothelial dysfunction due to exposure to cardiovascular risk factors might induce apoptosis and decrease...
the survival of recruited cells, and thus interfere with renal regeneration.

The cholesterol-lowering drugs 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors (statins) exert profound pleiotropic antiinflammatory, antiproliferative, and immunomodulatory actions, which may partly account for their beneficial effects on renal function and structure in experimental models of renal disease.18–20 Furthermore, atorvastatin has recently been shown to improve the myocardial microenvironment and facilitate mesenchymal stem cell survival and differentiation during acute infarction and reperfusion.21 However, the extent of EPC recruitment or fate in the kidney during exposure to HT and the efficacy of statins to improve renal repair have not been fully explored.

Therefore, the current study was designed to test the hypothesis that simvastatin improves the survival of EPC in the renal microenvironment imposed by HTC, in association with improved renal function and fibrosis.

Methods

The Institutional Animal Care and Use Committee approved the procedures. Seventeen domestic pigs were studied after 12 weeks of observation. Eleven pigs (50 to 60 kg) were studied 12 weeks after induction of unilateral renal artery stenosis and renovascular HTN, which was accompanied by a high-cholesterol diet of 2% cholesterol and 15% lard (TD-93296, Harlan Teklad)22 initiated on the same day. Renal artery stenosis was induced by coil implantation in the renal artery, which leads to development of HTN within 5 to 10 days.10,23,24 Of these 11 pigs, 5 also received chronic supplementation with simvastatin (1.2 mg/kg per day, HTC S) at a dosage that is used clinically (up to 80 mg/day) but has lower efficacy and does not decrease lipid levels significantly in pigs.25,26 Six age-matched untreated pigs served as normal controls. As we have done before, we secured a femoral catheter in the femoral artery for monitoring daily blood pressure until the end of the study.10,19,23,24

After 12 weeks of observation, in vivo electron beam computed tomography studies were performed for assessment of basal renal blood flow (RBF) and glomerular filtration rate (GFR) of the nonstenotic kidney, and they were repeated during suprarenal infusion of acetylcholine (Ach) to assess endothelial function.19 Blood samples were collected from the inferior vena cava for measurement of lipid profile (Roche Laboratories, Basel, Switzerland), creatinine (in the clinical laboratory), circulating oxidized low-density lipoprotein (ox-LDL) (Mercodia, Uppsala, Sweden), and plasma renin activity (radioimmunoassay) and from the renal vein of the HTC or normal kidney for measurement of plasma concentration of basic fibroblast growth factor (bFGF) using enzyme immunoassay,26 as described previously.27 EPC homing signals were assessed by SDF-1 expression in the kidney. Urine samples were collected at the end of the study using a suprapubic puncture, and protein concentration was determined as described previously.17 After completion of all studies, the pigs were euthanized with a lethal injection of Sleepaway (100 mg/kg intravenously; Ft Dodge Laboratories, Inc, Ft Dodge, Iowa). Kidneys were removed using a retroperitoneal incision and immediately dissected, and sections were shock-frozen in liquid nitrogen (and maintained at −80°C) or preserved in formalin.24 Fixed blocks of tissue were embedded in paraffin, and 5-μm-thick sections were cut from each block. Capillaries were manually counted using hematoxylin/eosin staining, as described previously.28 Furthermore, kidney sections were stained for angiomodulin, a protein that is often upregulated in angiogenesis.28

Harvested renal tissue (cortex and medulla) was then used for in vitro studies. Renal EPC recruitment was evaluated by double immunostaining of CD34 with vascular endothelial growth factor (VEGF), KDR (VEGFR receptor-2), or tumor necrosis factor-α (TNF-α) to test for EPC phenotype and paracrine function. Apoptosis was evaluated by the terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) assay, activated caspase-3 staining, and protein expression of the proapoptotic apoptosis-inducible factor and antiapoptotic Bcl-xL. To identify the apoptotic cells, concurrent TUNEL and staining for macrophage, fibroblast, tubular cell (cytokeratin), renal resident stem cell (Oct-4), or EPC markers were performed. To assess the renal microenvironment, oxidative stress was evaluated by the expression of the NADPH oxidase (p47phox and p67phox subunits) and in situ production of superoxide anion detected by fluorescence microscopy using dihydroethidium, as previously described.29 Renal inflammation was evaluated by expression of mononuclear chemoattractant protein and macrophage infiltration. Fibrosis was assessed by trichrome staining of kidney tissue and by bFGF levels in the renal vein. For details, see the online supplement, available at http://atvb.ahajournals.org.

To explore the possible mechanisms involved in the effects of simvastatin on EPC, peripheral blood was also collected from HTC pigs for isolation and culture of EPC, using a standard protocol as we described previously.30 EPC were then exposed to the proapoptotic factor ox-LDL or TNF-α, cocultured with or without simvastatin, and EPC apoptosis, proliferation, and migration were evaluated. The expression of CHOP, a protein that mediates endoplasmic reticulum stress-induced apoptosis,30 was also assessed. For details, see the online supplement.

Statistical Analysis

Results are expressed as mean±SEM. Comparisons within groups were performed using the paired Student t test and among groups using ANOVA, followed by the Tukey test. Statistical significance for all tests was judged at P<0.05.

Results

General Group Characteristics

Mean arterial pressure, total cholesterol, and low-density lipoprotein cholesterol were all significantly and similarly increased in HTC and HTC+S compared with normal (Table). Plasma creatinine and urine protein levels remained similar among the groups (Table). Renal vascular resistance was increased in HT and slightly attenuated in the HTC+S group.

| Table. Characteristics of Normal, HTC, and HTC+S Pigs |
|----------------------|----------------------|----------------------|
|                     | Normal (n=6) | HTC (n=6) | HTC+S (n=5) |
| MAP, mm Hg         | 103±2.8       | 133.6±4.5*  | 134±4.2*   |
| Total cholesterol, mmol/L | 1.6±0.2  | 9.2±1*     | 8.28±1.8*  |
| LDL cholesterol, mmol/L | 0.8±0.1  | 7±1*       | 6.5±1.7*   |
| Ox-LDL, units/L    | 12.6±1.7      | 24.4±1.4*   | 18±2.6†    |
| PRA, ng/ml per hour| 0.18±0.06     | 0.21±0.09   | 0.26±0.07  |
| Uric acid, mg/dL   | 1.3±0.1       | 1.9±0.2*    | 1.4±0.1    |
| Creatinine, mg/dL  | 1.2±0.1       | 1.4±0.2     | 1.7±0.3    |
| Urine protein, μg/mL | 26.1±4.1     | 23.1±4.3    | 28.4±1.4   |
| bFGF, pg/mL        | 8.5±1         | 14.8±1.6*   | 9.4±1.42†  |
| Renal volume, cm³  | 142±6.2       | 173.7±6*    | 173±4*     |
| RBF, mL/min        | 590±15        | 670±11*     | 639±11*    |
| RVR, mm Hg/mL per min | 0.19±0.04  | 0.24±0.05*  | 0.21±0.05  |
| GFR, mL/min        | 715±2.4       | 92±6.4*     | 91.2±4*    |

Uric acid and bFGF were measured in the renal vein. LDL indicates low-density lipoprotein; PRA, plasma renin activity; RVR, renal vascular resistance.

*P<0.05 compared with normal.
†P<0.05 compared with HTC.
whereas plasma renin activity was not different among the groups (Table).

Renal Hemodynamics and Function

Basal renal volume, RBF, and GFR were all significantly elevated in HTC and HTC/H11001S compared with normal. However, the increase of RBF and GFR from baseline in response to Ach was significantly blunted in HTC compared with normal, and it was improved in HTC+S (Figure 1).

**HTC Induces Oxidative Stress and Inflammation, Which Are Attenuated by Simvastatin**

Ox-LDL was elevated in the HTC group but reduced (although not normalized) in HTC+S (Table). Dihydroethidium staining showed an increased abundance of superoxide anion in HTC compared with normal (7.4±1.6% versus 1.8±0.8%, P<0.05); this increase was attenuated by simvastatin (to 2.6±0.4%, P<0.05 versus HTC) (Figure 2). The expression of p47 (NAD(P)H oxidase subunit) was also upregulated in HTC and normalized by simvastatin, although p67 remained elevated in HTC+S kidneys (Figure 2). The expression of mononuclear chemoattractant protein, an important regulator of macrophage recruitment, was also increased in HTC and normalized in HTC+S (Figure 3), suggesting decreased inflammation by simvastatin. Furthermore, the number of interstitial macrophages was significantly increased in HTC compared with normal (12.3±1.3 versus 1.0±0.2 per field, P<0.001), but it was significantly decreased by simvastatin (2.7±0.8 per field, P<0.05 versus HTC, P=0.09 versus normal) (Figure 3).

**HTC Elicits EPC Apoptosis, Which Is Blunted by Simvastatin**

The number of renal CD34+/KDR+ cells was increased in both HTC and HTC+S compared with normal kidneys, suggesting recruitment of circulating EPC (Figure 4). The CD34+ cells were concentrated mostly in the medullary area. Importantly, CD34+ cells were observed adjacent to increased expression of both VEGF and TNF-α in HTC (Supplemental Figure I).
Interestingly, simvastatin further increased VEGF expression but had no effect on TNF-α (Supplemental Figure I). In addition, SDF-1 expression increased in both HTC and HTC+S kidneys, whereas the increased renal vein levels of the homing signals SCF and uric acid in HTC returned to normal level in HTC+S (Figure 4, Table). The number of TUNEL+ and caspase-3+ apoptotic cells was increased in HTC compared with normal kidneys; this increase was observed mainly in the medullary tubulointerstitial compartment, but it was significantly decreased by simvastatin (Figure 5), suggesting blunted apoptosis. Interstitial TUNEL+ cells did not costain with either macrophage (CD163), fibroblast (vimentin and smooth muscle actin; data not...
shown), or resident stem cell (Oct-4) markers, and very few coexpressed cytokeratin (Supplemental Figure II), but many costained with CD34 (Figure 5). Indeed, the number of CD34+/TUNEL+ cells increased in HTC, suggesting EPC apoptosis, which was attenuated by simvastatin (Figure 5). The number of Oct-4+ cells was unchanged in HTC compared with normal (0.08±0.02% versus 0.06±0.02%, \( P>0.05 \)) and was unaffected by simvastatin (0.08±0.03%), and they did not costain with TUNEL, indicating that resident stem cells may not be involved in renal fibrosis in HTC. The expression of the proapoptotic factor apoptosis-inducible factor remained unchanged among the groups, but that of the apoptosis inhibitor Bcl-xL was decreased in HTC and increased in HTC+S (Figure 3).

Histological analysis showed that capillary density was decreased in HTC compared with normal and improved in HTC+S kidneys (Supplemental Figure III). Furthermore, angiomodulin expression was also downregulated in HTC and improved by simvastatin, indicating that angiogenic activity was impaired in HTC and improved by simvastatin (Supplemental Figure III).

**Simvastatin Directly Attenuates EPC Apoptosis In Vitro**

Studies in isolated EPC showed that simvastatin significantly decreased ox-LDL-induced apoptosis in these cells, an effect that was inhibited by mevalonate. These data indicate that simvastatin directly reduced EPC apoptosis. Simvastatin achieved an antiapoptotic effect similar to that of tempol to attenuate ox-LDL-induced EPC apoptosis, but it showed less efficacy to prevent TNF-\( \alpha \)-induced EPC apoptosis (\( P=0.07 \) versus TNF-\( \alpha \) alone, Supplemental Figure IV). Interestingly, simvastatin downregulated the expression of CHOP, an important apoptosis-regulating protein, under conditions of endoplasmic reticulum stress. Thus, the antioxidant property of simvastatin may contribute more than its antiinflammation property to decrease apoptosis in EPC. Ox-LDL also impaired EPC migra-
tion and proliferation, both of which were normalized by simvastatin (Supplemental Figure II).

**HTC Induces Renal Fibrosis, Which Is Attenuated by Simvastatin**

The levels of bFGF renal vein plasma were elevated in HTC compared with normal kidney, and they were normalized by simvastatin (Table). Renal trichrome staining showed increased interstitial and perivascular fibrosis in HTC compared with normal kidney (3.8±0.5% versus 0.36±0.05%, P=0.001), and it was attenuated in HTC+S kidneys (1.3±0.1%, P=0.005, versus HTC and normal, Figure 2). Minimal tubular atrophy was observed in HTC animals, but no glomerulosclerosis or tubular epithelial cells expressing vimentin or α-smooth muscle actin (data not shown) were observed in any group.

**Discussion**

This study shows that HTC in swine is associated with recruitment of EPC to the kidney, but at the same time, it is associated with increased EPC apoptosis, possibly because of renal endothelial dysfunction, oxidative stress, and inflammation, which are associated with renal interstitial fibrosis. Chronic simvastatin supplementation improved the renal microenvironment and decreased apoptosis, suggesting improved EPC survival. These pleiotropic effects were conferred without affecting blood pressure, cholesterol levels, or basal renal function, suggesting a role for simvastatin in protecting the kidney during exposure to concurrent HC and HTN, independent of systemic effects. Our in vitro data confirmed that simvastatin directly decreased ox-LDL-induced EPC apoptosis.

CKD has become an important public health concern in the United States and constitutes a risk factor for end-stage renal disease, cardiovascular and cerebrovascular disease, and premature death. The evolution of CKD and its cardiovascular outcomes are accelerated and aggravated by clustering of risk factors, such as HTN and HC. Indeed, it has become clear that the most effective approach to preserve renal function and slow down the consequences of CKD is to identify and treat risk factors and renal injury at the early stage of CKD.

In our HTC model, renal injury was evoked by a short coexistence of HTN and HC, which are often observed in a clinical setting. HTN can injure endothelial, mesangial, tubular, and interstitial cells and may cause local inflammation, oxidative stress, and fibroproliferative response. HC leads to deposition of oxidized lipids, decreased nitric oxide bioavailability, endothelial dysfunction, inflammation, and fibrosis. Both oxidative stress and inflammation can evoke apoptosis of renal cells and EPC. Bone marrow–derived CD34+ EPC participate in tissue repair in many diseases, and an imbalance of kidney damage and repair may lead to dysfunction and fibrosis of the affected kidney. Indeed, our study demonstrated that the pro-oxidant and inflammatory milieu induced by HTC is accompanied by apoptosis mainly of interstitial CD34+/KDR+ progenitor cells, presumably recruited to participate in kidney repair, thereby impairing renal self-regeneration potential. Notably, the HTC kidney also exhibited a small number of apoptotic tubular cells, but no apoptotic cells costaining with CD163, vimentin, α-smooth muscle actin, or Oct-4. Furthermore, the unchanged number of Oct-4+ cells in HTC animals indicates that resident stem cell may not be involved in renal fibrosis in HTC. Interestingly, the medulla recruited most of the EPC, and most EPC apoptosis was also observed in the medulla. The medulla of the kidney is considered perpetually hypoxic even under physiological conditions because of its unique circulation, and it is vulnerable to insults. A previous study also suggested that acute renal ischemia induces tubular cell apoptosis and macrophage infiltration in the medulla. It is not unlikely that similar susceptibility drives EPC recruitment to the noxious microenvironment secondary to hypertension and HC.

In HTC, administration of simvastatin may help the kidney restore the balance of injury and repair through several mechanisms. We have shown that simvastatin attenuated coronary and renal inflammation and oxidative stress, and improved endothelial function in HC. This study extended our previous observations by showing a potential role for simvastatin in attenuating kidney injury by decreasing circulating ox-LDL, downregulating p47phox, and attenuating dihydroethidium staining in HTC pigs. Furthermore, simvastatin blunted the upregulated mononuclear chemoattractant protein expression and macrophage infiltration in HTC, suggesting decreased local inflammation. It is pertinent that both decreased oxidative stress and inflammation may have contributed to blunt apoptosis of EPC participating in kidney repair. Our results show increases in both EPC (CD34+/KDR+ cells) and apoptotic EPC (TUNEL+/CD34+) numbers in HTC, and they show that simvastatin decreased only TUNEL+/CD34+ apoptotic cells, not overall EPC numbers, indicating that simvastatin decreased EPC apoptosis in HTC. We also found that SDF-1, a major EPC homing factor, was similarly increased in HTC and HTC+S pigs. These may have resulted in a similar number of CD34+ cells in HTC and HTC+S. It is possible that at this early stage of the disease, EPC recruitment signaling secondary to renal injury is still intact, yet the noxious microenvironment leads to their apoptosis and thereby decreases their availability. The observation that the number of EPC in the HTC+S kidney was similar to that in HTC kidney, in the face of markedly decreased renal injury, may, in fact, imply that simvastatin increases EPC mobilization despite the decrease in renal damage.

The decreased expression of the antiapoptotic factor Bel-xL and activation of caspase-3 in HTC might have facilitated EPC apoptosis, but the proapoptotic factor apoptosis-inducible factor remained unchanged among the groups, suggesting a direct role for additional mediators, such as increased oxidative stress and inflammation, in promoting EPC apoptosis in HTC. Our in vitro data also showed that ox-LDL increased in EPC expression of CHOP, a protein that mediates apoptosis induced by endoplasmic reticulum stress, implicating it in regulation of EPC apoptosis. These results agree with previous studies showing that cardiovascular risk factors elicit endoplasmic reticulum stress that can be ameliorated by statins. Simvastatin downregulated ox-LDL-induced CHOP expression in a mevalonate-independent manner, suggesting that it blunts endoplasmic
reticulum stress via an alternative pathway. Furthermore, we found that simvastatin rescued EPC migration and proliferation that were attenuated by ox-LDL in vitro. The increased spatial association with VEGF in the HTC+S kidney may imply improved EPC paracrine function as well, whereas cytokine expression (TNF-α) was unaffected. Our findings are underscored by previous studies showing that statins promote differentiation of mononuclear cells to EPC in vitro,\(^46\) enhance progenitor cell migration and proliferation, and downregulate EPC apoptosis in the ischemic hindlimb.\(^47\) Simvastatin had no effect on apoptosis in untreated EPC, but it blunted ox-LDL-induced EPC apoptosis to an extent similar to the superoxide dismutase mimic antioxidant tempol. On the other hand, it was less effective in preventing TNF-α-induced EPC apoptosis. Thus, the antioxidant property of simvastatin may contribute more than its antiinflammation trait to attenuate EPC apoptosis. SDF-1, SCF, and uric acid may have signaled EPC homing to the kidney, because their levels were increased in the HTC kidney or vein. A decrease in renal vein SCF and uric acid by simvastatin may reflect blunted renal injury, as well as attenuated oxidative stress (because uric acid is a downstream product of xanthine oxidase).

In this study, simvastatin supplementation did not reduce the elevated blood pressure (the driving force of renal perfusion pressure), and the baseline function of the HTC kidney therefore remained greater than normal. Nevertheless, the elevated renal vascular resistance in HTC was slightly decreased with simvastatin, suggesting decreased microvascular tone. Importantly, simvastatin improved RBF and GFR response to Ach (reflecting improved endothelial function), possibly by increasing nitric oxide availability, an effect that may also contribute to dampening fibrosis.\(^48\) Furthermore, impaired GFR responses to challenge may accompany and be an index of early tubular dysfunction,\(^10\) which precedes alterations in traditional renal function indices (eg, plasma creatinine and urinary protein). Indeed, we have previously shown that functional impairment in GFR was associated not with glomerulosclerosis but with tubular dysfunction\(^10\) and that tubular dysfunction preceded structural injury.\(^49\) The improvement in the renal microenvironment (decreased inflammation and oxidative stress, improved endothelial function) and cellular survival and repair culminated in decreased fibrosis and attenuated renal injury.

Thus, simvastatin attenuated not only the structural but also the functional adverse effects of HTN and HC on the kidney. Because it lowered neither cholesterol levels nor blood pressure, this was likely a direct effect of the drug on pathogenetic mechanisms within the kidney. Indeed, statins at high doses improve renal function in normcholesterolemic animal models, and we have shown their efficacy in the ischemic swine kidney.\(^19\) The present study extends these observations and demonstrates new mechanisms for their capacity to protect the kidney exposed to a cluster of cardiovascular risk factors, using a large animal model and a clinically relevant dose, independent of lipid- or blood pressure–lowering properties.

**Limitations**

In the present study, simvastatin was administered as a preventive measure, and the pigs in our study were relatively young. It is not unlikely that additional mediators contribute to renal damage and repair during more severe and prolonged elevations in blood pressure or serum cholesterol, some of which may not be adequately blunted by statins. Additional studies are needed to examine the efficacy of simvastatin to reverse preestablished or long-duration renal damage, especially once glomerulosclerosis has developed. The drug is also less efficacious in pigs than in humans, and a higher dose is required to decrease cholesterol levels in this model, thus allowing demonstration of its pleiotropic effects. The effects of statins on blood pressure are inconsistent and were not observed in our study.

In summary, this study shows that at the early phase, the coexistence of cardiovascular risk factors HTN and HC impairs renal function and increases tissue oxidative stress and inflammation. Although this injury triggered EPC homing signals and recruitment, the noxious microenvironment led to their apoptosis, thereby perpetuating the imbalance of tissue damage and repair. Simvastatin improved the renal microenvironment and blunted EPC apoptosis and renal interstitial fibrosis. Our study suggests that simvastatin can modulate renal deterioration induced by HTN and HC and suggests a role for this drug in protecting the kidney.

**Sources of Funding**

This study was partly supported by NIH grants DK073608, HL77131, DK077013, and HL085307 and by an unrestricted Medical School Grant from Merck Pharmaceuticals.

**Disclosures**

None.

**References**


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Arterioscler Thromb Vasc Biol. published online March 4, 2010;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Supplemental Material

Online supplemental Methods

In vivo studies

For the in vivo studies, pigs were anesthetized with telazol (5 mg/kg intramuscular) and xylazine (2 mg/kg), 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, catheters were placed in the superior vena cava for contrast media injections, and in the abdominal aorta for drug infusion. EBCT flow studies were then performed, as previously detailed \(^{1-3}\), for assessment of basal regional-renal volume, RBF, and GFR. Briefly, 40 consecutive scans were sequentially acquired for this purpose after a central venous injection of the contrast medium iopamidol (0.5 ml/kg over 2 s). Scanning was then repeated during a 15-min suprarenal infusion of the endothelium-dependent vasodilator acetylcholine (Ach, 5 µg/kg per min), to test intra-renal microvascular endothelial function.

For data analysis, changes of density over time were sampled in manually traced regions of interest selected in EBCT images in the aorta, renal cortex, and medulla of the HTC kidney. Time-density curves were fitted with extended gamma-variate curve-fits, and used to calculate renal single-kidney GFR, and RBF, using previously validated methods\(^ {1-5}\).

In vitro Studies

Histology
Corticomedullary midhilar 5 µm cross-sections of the HTC or normal kidney (1 per animal) were examined for glomerulosclerosis, tubular changes, and fibrosis. In addition, using a computer-aided image-analysis program (MetaMorph®; Meta Imaging Series 6.3.2 Downingtown, PA, USA), trichrome staining was semi-automatically quantified in 15–20 fields of each representative slide by the computer program, expressed as percentage of staining of total surface area, and the results from all fields averaged 1-3.

**Dihydroethidium (DHE)**

In situ production of superoxide anion was measured in 30-µm frozen sections incubated for 30 minutes with DHE (2 µmol/L) in a light-protected humidified chamber at 37ºC. Cytosolic DHE exhibits blue fluorescence, but once oxidized by superoxide to ethidium bromide it intercalates within the cell's DNA, staining its nucleus a fluorescent red. Sections were examined under inverted fluorescence microscope and red fluorescent was quantified using MetaMorph® program and expressed as percentage of staining of total surface area 6.

**Western blotting**

Standard blotting protocols were followed, as described previously 7, using antibodies against AIF (BD Bio Science, CA, 1:1000), Bcl-xL (Santa Cruz Biotechnology, Inc., CA; 1:200), MCP-1 (Biovision, CA, 1:500), SDF-1 (1:750, Abcam plc), and NAD(P)H oxidase subunits p47phox and p67phox (Santa Cruz Biotechnology, Inc., CA; 1:200), while β-actins (Sigma, 1:500) were used as loading controls. The intensities of the renal protein bands (one per animal) were quantified using densitometry,
normalized for loading controls, and averaged in each group. Plasma albumin was filtrated for SCF Western blotting, and the results were normalized using total protein loading on the membrane visualized by Ponceau S solution (Sigma).

**TUNEL and immunohistochemistry**

Apoptosis of kidney cells was evaluated in paraffin-embedded (5 μm thick) renal sections stained using apoptosis detection kit (Promega, WI), following manufacturer’s instructions, as well as by immunoreactivity of activated caspase-3 (1:20, Santa Cruz Biotechnology, Inc., CA), a major mediator of cell apoptosis. To determine apoptotic cell type, immunostaining of the fibroblast markers vimentin (1:50, Santa Cruz, CA) and α-smooth muscle actin (SMA, 1:50, Dako, Demark), and the macrophage (CD163, 1:50, AbD Serotec, Oxford, UK), progenitor cell CD34 (1:100, R&D System, MN), or resident progenitor cell Oct-4 (1:50, Lifespan Biosciences, Seattle, WA) markers were performed on the same slides the following day after TUNEL. Double immunofluorescence staining for CD34 (1:50, R&D System, MN) and KDR (1:20, Santa Cruz Biotechnology, Inc., CA), VEGF or TNF-α (1:20, Santa Cruz Biotechnology, Inc., CA) were performed using standard protocol to verify the endothelial phenotype and paracrine function of the EPC. Angiomodulin (1:500, Abcom) staining was performed in paraffin sections using standard protocol. To quantify the frequencies of apoptotic cells and EPC, two-color analyses were performed using a confocal microscope (Zeiss LSM 510, Germany). Ten random fields were captured from each kidney, and total apoptotic cells, TUNEL/CD34 double positive cells, and CD34/KDR double positive cells were quantified as the area of positive staining divided by the total area in each field using MetaMorph program, as we previously reported7.
Effects of simvastatin on EPC apoptosis, migration, and proliferation in vitro

EPC were isolated from peripheral blood of HTC pigs as we previously described\(^8\), and studied in vitro after exposure to the pro-apoptotic ox-LDL (50µg/ml)\(^9,10\) or TNF-\(\alpha\) (1 \(\mu\)mol/l), with or without co-incubation with simvastatin (1 \(\mu\)mol/l), mevalonate (500µmol/L), or the SOD mimic tempol (1mmol/L) for 12 hrs. Apoptosis was evaluated using APOPercentage™ kit (Biocolor Ltd, UK), following the manufacturer’s instruction. Briefly, after treatment EPC were incubated with the APOPercentage dye for 30min, washed twice, then the cell bound dye was removed into dye release reagent and absorbance measured at 550nm.

EPC migratory function was examined using a modified Boyden chamber technique (QCM Haptotaxis Cell migration assay, Millipore), following the manufacturer’s instructions\(^11\). EPC proliferative activity was determined by a tetrazolium compound MTS assay (CellTiter 96 Non-Radioactive Cell Proliferation Assay; Promega, Madison, WI), which monitors the number of viable cells, as we have previously shown\(^11\).

In 4 groups of EPC (control, ox-LDL, ox-LDL with simvastatin (ox-LDL+S), and ox-LDL with simvastatin and mevalonate (ox-LDL+S+M)) CHOP (1:500, Thermo Scientific) expression was also evaluated using a standard Western Blotting protocol.

References


**Supplemental Figure legends**

**Figure I** Representative images of CD34/VEGF and CD34/TNF-alpha immunostaining, and quantification of double positive cells (yellow), in normal, HTC, and HTC+S pigs, showing increased VEGF and TNF-α expression adjacent to CD34+ cells in HTC and HTC+S animals. *p<0.05 vs. Normal, †p<0.05 vs. HTC

**Figure II** Top: Representative images of TUNEL with cytokeratin or Oct-4 co-immunostaining, showing very few TUNEL/cytokeratin or TUNEL/Oct-4 double positive cells in kidney sections. Bottom: In vitro experiments illustrating that EPC exposed to ox-LDL showed impaired migration and proliferation function, which were improved by simvastatin. *p<0.05 vs. control

**Figure III** Top: Representative images of angiomodulin immunostaining (brown) in normal, HTC, and HTC+S pigs. Bottom: quantification of angiomodulin immunostaining showing decreased angiomodulin expression in HTC, and improvement by simvastatin.
Furthermore, peritubular capillary density was also decreased in HTC and improved in HTC+S. *p<0.05 vs. Normal, †p<0.05 vs. HTC

**Figure IV** Top left: in vitro EPC apoptosis assay showing that both ox-LDL and TNF-alpha induce EPC apoptosis, and simvastatin or tempol similarly attenuated ox-LDL-induced EPC apoptosis. However, simvastatin has less efficacy to prevent TNF-alpha induced (p=0.07 vs. TNF-α) compared to ox-LDL-induced EPC apoptosis. Bottom: representative Western Blot bands and quantification of CHOP expression in EPC that were untreated (control), or treated with ox-LDL, ox-LDL+ simvastatin (S), or ox-LDL+S+ mevalonate (M). Increased CHOP expression in EPC treated with ox-LDL was prevented by simvastatin but unaffected by mevalonate. *p<0.05 vs. control, †p<0.05 vs. ox-LDL, ‡p<0.05 vs. TNF-α, &p=0.07 vs. TNF-α
Figure III

Renal angiomodulin expression (%)

Peritubular capillary density (number/tubule)
Figure IV

EPC apoptosis

A550

CHOP

GAPDH

EPC CHOP expression (relative to GAPDH)