Hypertension and Hypercholesterolemia Differentially Affect the Function and Structure of Pig Carotid Artery

Daniele Versari, Mario Gossl, Dallit Mannheim, Elena Daghini, Offer Galili, Claudio Napoli, Lilach O. Lerman, Amir Lerman

Abstract—The purpose of this work was to compare the effects of hypertension and hypercholesterolemia on carotid endothelial function, structure, and vasa vasorum density. Seventeen pigs were randomized to a 12-week normal diet without (n=5), or with renovascular hypertension (HT; n=6), or to a high cholesterol diet (HC; n=6). Carotid arteries were studied by organ chambers (endothelial function) and microcomputed tomography (vasa vasorum), and tissue was processed for Sirius red staining and immunoblotting (vascular endothelium growth factor, endostatin, matrix metalloproteinase-9, and matrix metalloproteinase-2). HC and HT showed reduced vasodilation to acetylcholine as compared with controls, but HT also had a lower response to sodium nitroprusside. In addition, HT showed a higher content of organized collagen fibers and increased intima-media thickness. Vasa vasorum density was increased in HC but not in HT. Both HT and HC showed a proangiogenic biochemical milieu (higher vascular endothelium growth factor, matrix metalloproteinases, and lower endostatin), but this was more pronounced in HC. Both hypertension and hypercholesterolemia induce endothelial dysfunction in the carotid artery. However, hypertension is also associated with greater fibrosis and vascular wall thickening, which might impair endothelium-independent vasorelaxation and vasa vasorum growth. Hypercholesterolemia is, in turn, associated with vasa vasorum neovascularization. These data suggest that carotid atherosclerosis can evolve through different mechanisms in relation to different risk factors. (Hypertension. 2007;50:1063-1068.)

Key Words: carotid artery ■ atherosclerosis ■ hypertension ■ hypercholesterolemia ■ vasa vasorum ■ endothelium

Stroke is a leading cause of death in Western countries, and in the majority of the cases it is the consequence of an acute complication of an atherosclerotic carotid plaque. Risk factors for carotid atherosclerosis include the traditional cardiovascular risk factors, and, in particular, hypertension plays a major role. Although in the past hypercholesterolemia was not considered a determinant contributor to cerebrovascular disease, recent epidemiological studies and clinical trials with lipid-lowering drugs demonstrated a clear relation between serum cholesterol levels and carotid atherosclerosis, as well as with the risk of stroke.

Both hypertension and hypercholesterolemia are characterized by similar proatherogenic hallmarks, including endothelial dysfunction, vascular inflammation, and oxidative stress. However, the consequences of the action of these 2 risk factors on the arterial wall might be substantially different. Indeed, different cardiovascular risk factors influence atherosclerosis development favoring preferential plaque characteristics, eventually leading to the formation of morphologically different lesions. Experimental analysis of the interaction between hypertension and hypercholesterolemia on the mechanisms of early atherosclerosis indicates that hypertension, per se, induces adaptive remodeling, whereas for the maladaptive (atherogenic) intima-media thickening, hypercholesterolemia might be necessary.

The vessel wall thickening leads to a progressive ischemia within the arterial wall that can, in turn, contribute to the activation of proatherosclerotic mechanisms and, consequently, to plaque formation and progression. Furthermore, the relative ischemia of the arterial wall can activate compensatory neovascularization within the vasa vasorum (VV) system to normalize oxygen supply, mainly through the stimulation of hypoxia-inducible factor (HIF)-1α, which, in turn, increases vascular endothelial growth factor (VEGF). This proangiogenic pathway can also be enhanced by the increase of vascular oxidative stress, which upregulates HIF-1α, and by vascular inflammation associated with hypertension and hypercholesterolemia. Antiangiogenic factors, such as endostatin, and matrix remodeling enzymes, such as matrix metalloproteinases...
(MMPs), are also important systems in the regulation of the angiogenetic process. Despite being a compensatory mechanism, VV neoangiogenesis might eventually contribute to plaque progression and, in the late phases of atherosclerosis, to its destabilization by facilitating the recruitment of circulating precursors of inflammatory cells, intraplaque hemorrhage, and thrombosis. We have demonstrated previously that the VV neovascularization can even precede the development of endothelial dysfunction, which is generally considered the earliest stage of atherosclerosis.

In humans it is difficult to differentially study the effect of hypertension and hypercholesterolemia on the early phases of atherosclerosis development, because they are frequently associated in clinical practice, cluster with other risk factors, and are recognized in later phases. Therefore, we designed the present study to test the hypothesis that in the early phases of carotid atherosclerosis, hypertension, and hypercholesterolemia might differentially affect arterial function and structure. In particular, we evaluated the effect of these risk factors on endothelial function, local and systemic oxidative stress, carotid structure, angiogenetic pathway, and VV neovascularization in porcine experimental models of hypertension and hypercholesterolemia.

Materials and Methods
Seventeen female cross-bred domestic pigs, 3 months of age, were randomized to be fed a normal diet ad libitum for 12 months, without or with hypertension induced by placement of a local irritant stent in the left renal artery (groups N, n = 5, and HT, n = 6, respectively) as described previously, or a high-cholesterol diet (group HC; n = 6). At the end of the study, blood samples were collected for assessment of plasma lipid and oxidative stress parameters. Animals were then euthanized, and the vascular block, including the aortic arch and removal of vascular specimens, 1 internal carotid artery was processed for microcomputed tomography analysis of VV architecture. All of the procedures respected the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Mayo Foundation Institutional Animal Care and Use Committee. For details, please see the data supplement available at http://hyper.ahajournals.org.

Results
After a 12-week follow-up, the 3 groups showed similar body weight (N: 57.8 ± 1.5 kg; HC: 59.4 ± 2.4 kg; HT: 56.0 ± 3.5 kg). HT had higher systolic, diastolic, and mean arterial pressure values (140 ± 4/104 ± 4/124 ± 4 mm Hg) as compared with N (125 ± 3/91 ± 3/114 ± 3 mm Hg; P < 0.01) and HC (124 ± 3/90 ± 2/113 ± 3 mm Hg; P < 0.01). Compared with N and HT, HC had significantly (P < 0.001) higher serum levels of total (N: 1.89 ± 0.13; HC: 0.81 ± 0.85; HT: 1.92 ± 0.15 mmol/L), high-density lipoprotein (N: 0.83 ± 0.08; HC: 2.54 ± 0.21; HT: 0.78 ± 0.10 mmol/L) and low-density lipoprotein (LDL) cholesterol (N: 0.91 ± 0.13; HC: 7.05 ± 0.75; HT: 1.01 ± 0.10 mmol/L).

Carotid Endothelial Function
Maximal vasorelaxation to acetylcholine was significantly lower in HT (18.4 ± 5.7%) and HC (13.7 ± 4.5%) as compared with N (48.1 ± 6.1%; P < 0.001 for both), and no difference was observed between HC and HT (Figure S1A). Calcium ionophore induced a similar vasorelaxation in N (42.0 ± 0.8%) and HC (43.8 ± 1.0%), but did not affect HT (3.1 ± 0.91%; P < 0.001 versus N; Figure S1B). Moreover, HT were characterized by a significantly reduced response to sodium nitroprusside (maximal relaxation 61.6 ± 7.9%) as compared with N (85.1 ± 3.4%; P < 0.05), whereas the response to sodium nitroprusside in HC was similar to the response in N (82.6 ± 1.8; Figure S1C).

Systemic and Local Oxidative Stress
HC showed significantly higher levels of LDL malondialdehyde and LDL relative electrophoretic mobility and lower LDL lag time as compared with N and HT (Table). Plasma thiobarbituric acid reactive substance levels in both HC and HT were higher than in N (Table).

Dihydroethidium (DHE) staining of carotid arteries demonstrated an increased production of superoxide anion in specimens from HT and HC, particularly in the endothelial layer (percentage of intima DHE positive nuclei: N: 33.6 ± 7.1%; HC: 77.2 ± 7.9; HT: 71.4 ± 7.4; P < 0.01 for both HC and HT versus N; Figure 1).

Histology and Collagen Content
Elastic van Gieson staining demonstrated a significant increase in intima-media thickness in carotid arteries from HT (0.77 ± 0.06 mm) as compared with N (0.54 ± 0.04 mm; P < 0.05) and HC (0.59 ± 0.02 mm; P < 0.01; Figure 2). The groups had similar lumen diameters (N: 0.73 ± 0.09 mm; HC: 0.90 ± 0.10 mm; HT: 0.82 ± 0.05 mm; Figure 2). Sirius red staining (Figure 2) did not show any difference among the groups in the content of thinner collagen fibers in the media, whereas a nonsignificant tendency to an increase in thicker fibers was observed in HT. On the contrary, the adventitia of HT was characterized by a significantly decreased content of thinner collagen fibers and an increased content of thicker and organized fibers (Figure 2).

Carotid VV
Microtomographic morphometric parameters of carotid arteries from the 3 groups of pigs are shown in Table. Despite similar lumen areas, HT showed higher vessel wall areas as compared with N and HC. Moreover, whereas no difference was present between N and HT, HC were characterized by a significantly increased VV count, area, and spatial density (Table and Figure S2). No difference was observed in the average diameter of VV.

Angiogenesis Pathway
Immunoblotting analysis for VEGF demonstrated a significant increase in HC and a tendency to increase in HT as compared with N (Figure 3). Immunostaining confirmed the higher expression of VEGF and also demonstrated increased expression of HIF-1α in the outer media in both HC and HT (percentage of positive media nuclei: N: 10.7 ± 1.5%; HC: 30.7 ± 6.3%; HT: 45.9 ± 7.8%; P < 0.05 for HC and HT versus N; Figure 3). Moreover, immunoblotting showed lower expression of antiangiogenetic endostatin in both HC and HT.
Hypertension and hypercholesterolemia are well known to induce functional and structural alteration in the vessel wall, predisposing to atherosclerosis. Both risk factors are associated with increased systemic and vascular oxidative stress, leading to a reduced NO bioavailability and endothelial dysfunction. In the present study we confirm the presence of similarly reduced vasorelaxation to acetylcholine in HC and HT, associated with increased superoxide production in the endothelial layer and increased systemic oxidative stress. On the contrary, the response to calcium-ionophore was found impaired in HT, and was normal in HC, consistent with previous results in hypercholesterolemic animals and humans.

We found an inverse borderline significant correlation between red-orange staining with Sirius red and VV density ($r = -0.57; P = 0.08$). Correlations between other studied parameters were not statistically significant.

### Discussion

The present study demonstrates a differential effect of hypercholesterolemia and hypertension on porcine carotid artery function and structure in the early phases of atherosclerosis. In particular, both cardiovascular risk factors induce an increase in systemic and vascular oxidative stress, associated as expected with reduced vasorelaxation to endothelium-dependent stimuli. However, hypertension is also associated with a decrease in the response to endothelium-independent stimulus, increased vascular fibrosis, and media thickening as compared with N and HC. Moreover, only HC showed a significant increase in carotid artery VV density. These data suggest that, in the carotid artery, hypertension has a greater effect in inducing the early atherosclerotic structural hypertrophic modification as compared with hypercholesterolemia. This latter, on the other hand, induces VV neovascularization, which is potentially a crucial factor for the progression of the disease. The current study suggests that hypercholesterolemia and hypertension may favor the development of different functional and structural changes in the early phases of carotid atherosclerosis.

### Carotid Vascular Relaxation

Hypertension and hypercholesterolemia are well known to induce functional and structural alteration in the vessel wall, predisposing to atherosclerosis. Both risk factors are associated with increased systemic and vascular oxidative stress, leading to a reduced NO bioavailability and endothelial dysfunction. In the present study we confirm the presence of similarly reduced vasorelaxation to acetylcholine in HC and HT, associated with increased superoxide production in the endothelial layer and increased systemic oxidative stress. On the contrary, the response to calcium-ionophore was found impaired in HT, and was normal in HC, consistent with previous results in hypercholesterolemic animals and humans. Moreover, diversely from previous data in the pig coronary circulation, in HT a significant decreased response to sodium nitrouspnise was also detected, and this is consistent with that observed in the carotid district of rodent models of hypertension.

Although we cannot rule out the possibility of a reduced responsiveness of HT smooth muscle cells to the relaxant, the presence of structural modifications within the carotid arterial wall of HT seems to be the most probable mechanism, by restraining smooth muscle cells relaxation, because the vasorelaxation to sodium nitrouspnise is reversed by antifibrotic treatment. Conceivably, in our model, the observed increased intima-media thickness and the adventitial fibrosis contribute to the arterial stiffening and to the consequent impaired vasorelaxation. In particular, in the Sirius red staining, HT showed an increased signal in the spectrum of the orange-red wavelength, indicating accumulation of thick and more organized collagen fibers.

### Carotid VV

The presence of hypercholesterolemia has been demonstrated to stimulate VV neoangiogenesis in several animal models, and we demonstrated previously by microcomputed tomography the presence of VV within the carotid artery in normal pigs. The present study confirms the effect of
hypercholesterolemia in inducing VV neovascularization in the carotid district, conceivably through an activation of the HIF-1α/VEGF axis. This phenomenon in the early stages is potentially protective, protecting the vascular wall from the effects of a relative hypoxia, such as the increase in vascular oxidative stress, inflammation, and fibrosclerosis. In our study, the lack of arterial wall thickening in HC suggests that the main drive to vessel wall neovascularization might not be represented by local hypoxia but could rather be related to the increased oxidative stress, which is capable of activating HIF-1α.28

In the long term, the consequent increased exchange surface between the arterial wall and the circulating blood might conceivably favor the infiltration of lipids, oxidized lipids, inflammatory cells, and mediators, key mechanisms of atherosclerotic plaque formation.29 On the other hand, HT were characterized only by a smaller and nonsignificant increase in VV count as compared with N, in line with previous results in canine30 and porcine31 renovascular hypertension models. This, together with the significant increase in intima-media thickness, resulted in a VV density that was not different from N. Although in a previous work32 hypertensive rats were characterized by a significant increase in the number of aortic VV as compared with control animals, a parallel increase in vessel wall area was observed, conceivably determining nonchange in VV density. Partly in accordance with Kuwahara et al,32 we found an increased expression of HIF-1α and, to a lesser extent, of VEGF in carotid arteries from HT. We also observed an actual increase in VV count, that, however, was not significant. The present results suggest that, although in hypertension there is a drive toward neangiogenesis, as supported by the increased expression of HIF-1α, VEGF, MMPs, and the decreased expression of endostatin, the collagen accumulation and organization might limit the sprouting of new vessels. Moreover, although HT and HC showed a similar increase in the expression of HIF-1α, this was followed by a greater increase in VEGF in HC. Because HIF-1α is a major determinant for the proliferation of smooth muscle cells,33 it is possible that, in the presence of hypertension, characterized by increased wall stress, the HIF-1α downstream mediators tend to preferentially foster smooth muscle cell proliferation and vascular fibrosis to restore normal wall stress. On the contrary, the more favorable hemodynamics of HC allows the HIF-1α-VEGF pathway to promote neovessel sprouting.

The current study is consistent with what we found previously in the coronary circulation,25 showing a stronger drive toward vascular collagen accumulation in hypertension than in hypercholesterolemia. However, different from the coronary district,31 the hypertension-related carotid artery fibrosis results in an early impairment on the vascular wall distensibility and response to endothelium-independent stimuli. We can speculate that the elastic nature of the carotid artery tends to make it more prone to change its matrix structure by accumulating collagen. However, the clear mechanisms for the different responses of distinct vascular districts to cardiovascular risk factors are not known.
Implications for Atherosclerosis

It was demonstrated previously that the morphology of atherosclerotic plaques, which is a key determinant for the development of complications and clinical events, is differentially influenced by different cardiovascular risk factors; in particular, hypertension is more often associated with a granulomatous whereas hypercholesterolemia is associated with a xanthomatous plaque phenotype.

According to the response-to-injury theory of atherosclerosis, mechanical or chemical factors induce damage to the endothelium, followed by proliferation of smooth muscle cells, infiltration of leukocytes, and accumulation of oxidized lipids. Chobanian et al. showed that hypertension might represent one of the initiators of atherosclerosis, but it might not be able to induce the formation of atherosclerotic plaque without the presence of hypercholesterolemia. Indeed, although both cardiovascular risk factors are clinically associated with carotid intima-media thickening, hypertension, per se, is able to induce adaptive remodeling, but for the maladaptive (atherogenic) intima-media thickening, hypercholesterolemia might be necessary.

Hypertension and hypercholesterolemia may share similar proatherosclerotic mechanisms, such as vascular oxidative stress, endothelial dysfunction, and vascular inflammation; however, from the early phases, they seem to favor different functional and structural modifications in the vascular system. Mechanical stress, the related gene expression in hypertension, and the overload of native and oxidized forms of endogenous lipids in hypercholesterolemia are possible implicated differential mechanisms.

Although hypertension is the main risk factor for stroke, we can speculate that it is crucial for the initiation of the process by inducing endothelial dysfunction/damage and for the complication of the mature plaque by means of mechanical trauma. Hypercholesterolemia, in turn, through the related increased neovascularization of the arterial wall, might be fundamental for the further growth and development of the mature plaque; accordingly, hypercholesterolemia, but not hypertension, is associated with an unstable plaque phenotype.

A limitation of the present study is represented by the lack of a group of animals with both hypertension and hypercholesterolemia. Further studies analyzing the interaction of the 2 risk factors might be useful to understand the dynamics of carotid atherosclerosis and its later consequences. Moreover, the relatively small number of animals did not allow us to find significant correlations among the studied parameters. Finally, the observed effects relate to the experimental animal models used in the present study, and it is not clear whether they can be applied to other forms of hypertension and hypercholesterolemia. In particular, we used a model of renovascular hypertension in which the activation of the renin-angiotensin system might foster vascular fibrosis. It is possible that in other models of hypertension, such as aortic coarctation or in spontaneously hypertensive rats, these phenomena might be different for some aspects. Similar considerations are also valid for diet-induced hypercholesterolemia with respect to apolipoprotein E–deficient mice. However, although further studies are needed to confirm the present data, the porcine model reasonably resembles human vascular physiology and pathophysiology, and the experimental models that we used can be considered reliable surrogates of the early atherosclerotic process.

Perspectives

The present study confirms that hypertension and hypercholesterolemia represent noxious factors for the carotid circulation by promoting various mechanisms of the atherosclerotic process. However, the 2 risk factors are characterized by the development of partially different features of atherosclerosis with potentially different roles in the onset, progression, and complication of the disease. Future research should be directed to elucidate these aspects, which may have important clinical implications for the prevention and treatment of atherosclerosis.

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Disclosures

None.

References


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HYPERTENSION AND HYPERCHOLESTEROLEMIA DIFFERENTIALLY AFFECT THE
FUNCTION AND STRUCTURE OF PIG CAROTID ARTERY

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Animals

Seventeen female cross-bred domestic pigs, 3 months of age were randomized to be fed ad libitum a normal diet for 12 weeks, without (N; n=5) or with hypertension (HT; n=6) induced by placement of a local irritant stent in the left renal artery, as previously described\(^1\), or a high-cholesterol diet (HC; n=6) (2% cholesterol and 15% lard; TD 93296; Harlan Teklad). In previous works by our group, 12 weeks of hypertension or hypercholesterolemia, obtained by the above described procedures, have been found to induce significant changes in the function and structure of the animals’ heart, kidney and arteries, resembling corresponding human diseases\(^1\)\(^2\)-\(^6\).

At the end of the study, blood samples were collected for assessment of plasma lipid and oxidative stress parameters. Animals were then euthanized by an intravenous injection of a commercial euthanasia solution (10 mL Sleepaway; Ft Dodge Laboratories, Ft Dodge, Iowa). The vascular block including aortic arch, subclavian and carotid arteries was immediately harvested and placed into a cold modified Krebs-Ringer bicarbonate solution (118.3 mmol/l NaCl, 4.7 mmol/l KCl, 2.5 mmol/l CaCl\(_2\), 1.2 mmol/l MgSO\(_4\), 1.2 mmol/l KH\(_2\)PO\(_4\), 25 mmol/l NaHCO\(_3\), 0.026 mmol/l calcium EDTA, and 11.1 mmol/l glucose). After removal of vascular specimens, one of the two internal carotid arteries was processed for tissue analysis and endothelial function assessment; the rest of the block was processed for micro-CT study. All procedures respected the National Institutes of Health Guidelines and were approved by the Mayo Foundation Institutional Animal Care and Use Committee.

Induction of Renal Artery Stenosis

Animals were anesthetized with intramuscular ketamine (20 mg/kg; Fort Dodge Animal Health, Fort Dodge, Iowa) and xylazine (2 mg/kg; VET TEK, Blue Springs, Mo), intubated, and mechanically ventilated with room air. Anesthesia was maintained with constant infusion of ketamine (0.2 [mg · kg\(^{-1}\)/min] and xylazine (0.03 [mg · kg\(^{-1}\)/min]), which have minimal effects on renal hemodynamics in animal models. An intravenous bolus of heparin (5000 U) was followed
by continuous infusion (1000 U/h). With fluoroscopic guidance, a percutaneous transluminal balloon 7.0 mm in diameter (Cordis, Miami, Fla) wrapped with a copper coil (made in house from 23-gauge copper wire) was advanced through the femoral artery into the proximal middle section of the single left renal artery. The balloon was inflated twice to high pressure, deflated, and removed, leaving the local-irritant copper coil embedded in the vascular wall. Selective renal angiography was used to confirm vessel patency and coil location. It has been demonstrated that placement of such a copper stent in the renal artery leads to a gradual and progressive luminal narrowing (average, 65%–75%) within 10 days, associated with an increase in blood pressure values.

**In vitro analysis of vascular reactivity**

Carotid endothelium-dependent and –independent vasodilation were evaluated as previously described for coronary specimens. Briefly, carotid artery segments (2-3 mm long, 2-3 per animal) were dissected and transferred into an organ chamber with modified Krebs-Ringer bicarbonate solution and 94% O2, 6% CO2. The rings were mounted on a calibrated isometric force transducer for continuous recording of vessel wall tension. Viability of vessels was tested by the vasoconstriction response to 20 mmol/l KCl at baseline, 2, 4 and 6 g of wall tension. At 6 g wall tension vessels were challenged with the endothelial agonist substance P (10^{-6} mmol/l; Sigma, St. Louis, MO) in order to assess the functional integrity of the vascular endothelium. After washing with control solution and a resting period of 30 minutes, all the vessels rings were precontracted with 10^{-7} mol/l endothelin-1 (Phoenix Pharmaceuticals, Mountain View, CA) and challenged with vasoactive substances. Endothelium-dependent vasodilation was explored by increasing doses of the non receptor-operated vasodilator calcium ionophore A23187 (10^{-11} to 10^{-6} mol/L, Sigma), and the receptor-operated endothelium-dependent vasodilator acetylcholine (10^{-9} to 10^{-5} mol/L). Endothelium-independent vasorelaxation was assessed with a dose-response curve to sodium nitroprusside (10^{-9} to 10^{-4} mol/L).
Systemic oxidative stress

As systemic markers of oxidative stress, LDL oxidizability (as Lag time), LDL-MDA\textsuperscript{8}, LDL-REM\textsuperscript{8}, and TBARS\textsuperscript{9} were evaluated in plasma as previously described\textsuperscript{2,10}.

In situ detection of superoxide anion

In 30-µm frozen carotid artery sections, \textit{in situ} production of superoxide anion was assessed by the oxidative fluorescent dye dihydroethidium, as previously described\textsuperscript{10}. DHE appears with a blue fluorescence in the cytosol, but, when oxidized by superoxide to ethidium bromide, it intercalates in the cell's DNA, staining the nucleus a fluorescent red (excitation at 488 nm, emission 610 nm). Serial sections were equilibrated under identical conditions for 30 min at 37°C in Krebs-HEPES buffer. Fresh buffer containing 2 µmol/l DHE was applied onto each section, coverslipped, incubated for 30 min in a light-protected humidified chamber at 37°C, and then evaluated under fluorescence microscopy. Counterstaining with DAPI (Vector Laboratories, CA) was then applied and the percentage of DHE positive over the overall number of nuclei was calculated.

Microscopic Computed Tomography (micro-CT)

A plastic cannula was tied at the origin of the aortic arch and injected with 500 ml of heparinized saline (0.9% sodium chloride with 5,000 U of heparin) to clear the vascular lumen from the remaining blood. Next, a low-viscosity (20-centipoise) lead chromate–doped silicon polymer (Microfil\textsuperscript{TM}, MV-122; Canton Biomedical Products, Boulder, CO) was injected through the cannula at a constant rate of 2 mL/min. After overnight refrigeration at 4°C to allow polymerization of the compound, carotid arteries were carefully dissected and 2 segments (~2 cm long) were placed in a buffered formaldehyde solution for 3D Micro-CT imaging. The computerized reconstruction algorithm yielded an average of 500 slices per carotid artery segment with a matrix of 21-µm cubic voxels and a 16-bit gray scale. The reconstructed
images were analyzed using Analyze software (version 7.0; Biomedical Imaging Resource, Mayo Foundation, Rochester, MN). Cross-sections were obtained at 1-mm intervals, resulting, after cutting artifacts at the ends of the specimens, in 20-40 cross-sections. The study parameters, obtained\textsuperscript{4-6} were: vessel wall area, VV area, VV count, VV density (VV count / vessel wall area).

\textbf{Histology}

Fixed blocks of carotid tissue were embedded in paraffin, and 5-µm-thick sections were cut from each block. Elastic Van Gieson and hematoxylin and eosin staining were performed according to standard techniques and slides analyzed for morphometric assessment (MetaMorph, Meta Imaging series 4.6) of lumen area and external elastic lamina area to calculate intima-media area as (external elastic lamina area - lumen area)\textsuperscript{11}.

\textbf{Collagen content by Sirius red}

The interstitial collagen content of carotid arteries was evaluated by Sirius red, following standard procedures. Briefly, specimen sections were deparaffinized, rehydrated and incubated with 0.1% Sirius red in saturated picric acid for 60 minutes. After incubation in 1% acetic acid for 30 minutes and rinsing, slides were counterstained in hematoxylin, differentiated in acid alcohol solution, rehydrated and mounted. Slides were visualized under both bright-field and polarized light microscope, and pictures taken with identical exposure settings for all sections. The color of stained collagen under polarized light changes with the thickness of the fibers, from green to yellow, orange and red, and this latter represents more organized and stiff fibers\textsuperscript{12}. Images were analyzed by an image analysis software (MetaMorph, Meta Imaging Series 4.6) to semiautomatically quantify the percentage of artery area stained with green or orange-red color, indicating respectively thinner and thicker organized fibers\textsuperscript{12}. 

Western Blotting

As previously described\(^5,10,11\), equal protein amounts (150 µg) of carotid homogenate were resolved in SDS–polyacrylamide gels (8-15%) and electrophoretically transferred onto nitrocellulose membrane. After blocking for 1 hour in TBST/5% nonfat milk, membranes were incubated overnight at 4°C with primary antibodies: VEGF (1:200, Abcam, UK), endostatin (1:100, Oncogene, MA), MMP-2 (1:250, Chemicon International, CA), MMP-9 (1:250, Chemicon). Anti-mouse (Amersham Life Sciences, IL) or anti-rabbit (BD Pharmingen, NJ) horseradish peroxidase-conjugated secondary antibody (1:1000-1:10000) was then applied as appropriate. After washing, membranes were developed with chemiluminescence (Pierce, IL), and exposed to an X-ray film (Kodak, NY). After film development, optical density of immunoblots was quantified by ImageJ software (NIH). β-Actin (1:2500, Sigma) was used as the loading control.

Immunohistochemistry

After deparaffinizing and rehydrating, carotid slides were incubated overnight at 4°C with primary antibody anti-VEGF (1:500, Santa Cruz Biotechnology, CA) and HIF-1\(^\alpha\) (1:200, Santa Cruz). Subsequently appropriate secondary antibody was applied for 1 h at room temperature. Diaminobenzidine was used as chromogen and hematoxylin as counterstaining. Stained slides were then viewed under microscope (Olympus, Leeds Precision Instruments) and picture obtained and quantified by an imaging software (MetaMorph, Meta Imaging series 4.6).

Statistical Analysis

For the analysis of micro-CT data the parameters obtained in every 2-dimensional cross section were averaged for each pig. Continuous data are expressed as mean± SEM. Comparison of groups was performed using ANOVA or unpaired Student’s \(t\)-test. Statistical significance was accepted for \(p<0.05\).


Figure S1. Carotid artery vasorelaxation. Carotid artery vasorelaxation to different stimuli: receptor-operated endothelium-dependent acetylcholine (A), non-receptor-operated endothelium-dependent calcium ionophore (B), endothelium-independent sodium nitroprusside (C) in normal (n=5; black squares), hypercholesterolemic (n=6; black triangles) and hypertensive (n=6; black circles) pigs. *, p<0.05 N vs. HC and HT; †, p<0.05 HT vs. N and HC.
Figure S2. Carotid Artery Vasa Vasorum. Micro-CT visualization of carotid arteries after Microfill™ infusion and 3D rendering, showing higher content of vasa vasorum in HC (n=6) as compared to N (n=5) and HT (n=6) pigs.