Functional and structural remodeling of the myocardial microvasculature in early experimental hypertension

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Rodriguez-Porcel, Martin, Xiang-Yang Zhu, Alejandro R. Chade, Beatriz Amores-Arriaga, Noel M. Caplice, Erik L. Ritman, Amir Lerman, and Lilach O. Lerman. Functional and structural remodeling of the myocardial microvasculature in early experimental hypertension. Am J Physiol Heart Circ Physiol 290: H978–H984, 2006. First published October 7, 2005; doi:10.1152/ajpheart.00538.2005.—Advanced hypertension (HT), associated with left ventricular hypertrophy (LVH), impairs myocardial microvascular function and structure and leads to increased myocardial hypoxia and growth factor activation. However, the effect of HT on microvascular architecture and its relation to microvascular function, before the development of LVH (early HT), remains unclear. By way of method, pigs were studied after 12 wk of renovascular HT (n = 7) or control (n = 7) animals. Myocardial microvascular function (blood volume and blood flow at baseline and in response to adenosine) was assessed by using electron beam computed tomography (CT). Microvascular architecture was subsequently studied ex vivo using micro-CT, and microvessels (diameter, <500 μm) were counted in situ in three-dimensional images (40-μm on-a-side cubic voxels). Myocardial expression of vascular endothelial growth factor, basic fibroblast growth factor, and hypoxia-inducible factor-1α were also measured. By way of results, left ventricular muscle mass was similar between the groups. The blood volume response to intravenous adenosine was attenuated in HT animals compared with normal animals (246 vs. 125 vessels/cm2, P = 0.01, respectively). Microvascular spatial density in HT animals was significantly elevated compared with normal animals (246 ± 26 vs. 125 ± 20 vessels/cm2, P < 0.05) and correlated inversely with the blood volume response to adenosine. Growth factors expression was increased in HT animals compared with control animals. In conclusion, early HT elicits changes in myocardial microvascular architecture, which are associated with microvascular dysfunction and precede changes in muscle mass. These observations underscore the direct and early effects of HT on the myocardial vasculature.

microcirculation; imaging; growth factors; microcomputed tomography

HYPERTENSION (HT) is a major risk factor for the development and progression of ischemic heart disease and increases the incidence of cardiac events (13). Advanced HT leads to the development of left ventricular (LV) hypertrophy (LVH), which has been associated with abnormal myocardial microvascular function, myocardial architectural changes (9, 41), and myocardial ischemia (41, 44, 45). However, the effect of early HT, before the development of LVH, on the myocardial microvasculature (structure and function) is not well understood. We have recently shown that early HT was associated with an impairment in both coronary vascular response to vasodilators (35) in vitro and myocardial perfusion response to cardiac vasodilator challenge in vivo (31, 33). However, it is yet unknown whether these functional alterations are coupled with changes in myocardial microvascular architecture. Furthermore, the adequacy of such changes in the architecture of myocardial microvessels to sustain their function remains to be elucidated.

Remodeling of the myocardial microvascular architecture, like neovascularization, may represent an adaptive response of the coronary circulation to episodes of myocardial ischemia or sustained pressure overload (37) and is regulated by growth factors and their receptors acting in concert (14). In particular, basic FGF (bFGF) (5, 16) and VEGF (36), regulated through hypoxia-inducible factor (HIF)-1α (26), can mediate compensatory neovascularization, as described in the aortic vasa vasora in advanced HT (18) and in the coronary microvessels during the acute phase of adaptive cardiac hypertrophy (37). However, their role in early HT is yet to be determined.

One of the main factors that restricted investigation of the intramyocardial microcirculation is the limited availability of accurate and high-resolution imaging methods to study both myocardial microvascular function and three-dimensional (3-D) architecture in situ. Studies (32, 33) have previously shown that electron beam computed tomography (EBCT) allows accurate and noninvasive assessment of microvascular function in vivo, including myocardial perfusion, microvascular blood volume (BV), and permeability. In addition, microcomputed tomography (micro-CT) is a unique and high-resolution technique that allows investigation of small-scale structures within organs (12). Previous studies (2, 3, 34, 48) have shown that it permits assessment of the 3-D pattern of microvascular structure, spatial distribution, and connectivity. The combined application of these two powerful functional imaging techniques may therefore provide a unique insight into the concurrent functional and structural changes of the intramyocardial microcirculation at an early stage of adaptive cardiac hypertrophy (37).

Thus the present study was designed to test the hypothesis that early HT is associated with growth factor activation and changes in myocardial microvascular architecture, such as neovascularization, and, furthermore, that the increased spatial density of myocardial microvessels would correlate with the extent of their dysfunction in vivo (e.g., the degree of myocardial BV response to adenosine).
MATERIALS AND METHODS

All procedures were approved by the Institutional Animal Care and Use Committee. Female domestic pigs (55–65 kg, 6 mo of age) were studied after 12 wk of observation. Group 1 had no intervention (n = 7 pigs), whereas in group 2 renovascular HT (n = 7 pigs) was induced by placing a local irritant stent in the left renal artery with consequent renal artery stenosis and development of hypertension (21). After 12 wk, animals were studied in vivo and blood samples were collected for measurement of plasma renin activity (PRA). Animals were then euthanized with intravenous pentobarbital sodium (20 ml of Sleepaway, Fort Dodge Laboratories, Fort Dodge, IA), and hearts were immediately removed and prepared for micro-CT, as previously described (34, 48). In addition, transmural pieces of the myocardium were flash frozen in liquid nitrogen or preserved in formalin for measurement of growth factor expression.

LV muscle mass and microvascular function via EBCT. For in vivo studies, animals were anesthetized with ketamine and xylazine (20 mg/kg and 2 mg/kg, respectively), intubated, and mechanically ventilated. Catheters were placed fluoroscopically in the aorta for measurement of mean arterial pressure (MAP) and in the right atrium for contrast media injections. Animals were then placed in the EBCT-scanning gantry (32, 33).

To exclude LVH, LV muscle mass was then measured (33). After allowing 15 min for stabilization, end-diastolic tomographic scans (from LV apex to base; slice thickness of 7 mm) were acquired by using ECG triggering. Scans were obtained during continuous infusion of iopamidol-370 (0.5 ml/kg, Squibb, Princeton, NJ), which enables the distinguishing of the endocardial border from the contrast-filled LV cavity during subsequent image analysis. The endocardial and epicardial borders were then manually traced, and LV muscle mass was calculated as a product of myocardial muscle area, density, and slice thickness, as previously shown (33).

For assessment of myocardial vascular function, scanning was performed in the EBCT flow mode in which 40 consecutive end-diastolic scans were obtained over 40 s (at 1–3 heart beat intervals) after a 2-s injection of iopamidol-370 (0.3 ml/kg) into the right atrium. Scans were obtained at baseline and after a 10-min intravenous adenosine infusion (400 µg·kg⁻¹·min⁻¹). Intravenous adenosine induces coronary artery vasodilation and is the most common pharmacological cardiac challenge used to assess myocardial perfusion reserve in clinical practice.

For image analysis, regions of interest were obtained from the LV cavity (for input function) and the anterior cardiac wall. The average tissue density (CT numbers) during and consequent to transit of contrast media was then measured in each region of interest and plotted against time, yielding time-density curves. Each myocardial time-density curve was then stripped into two components, representing the transit of contrast media through the intravascular and extravascular compartments of the myocardium, using an extended gamma-variate, curve-fitting algorithms. CT, computed tomography.

artery (LAD) was cannulated and perfused with a solution of 0.9% normal saline and heparin, and an intravascular contrast agent, radiopaque microfil silicone rubber (MV-122, Flow Tech, Carver, MA), was perfused through the cannulated LAD at a flow rate of 0.9 ml/min (at a physiological perfusion pressure of 100 ml/min) until it flowed freely from the myocardial veins. To allow for complete relaxation, the hearts were kept at 4°C for a day (34), after which a transmural portion of the LV myocardium (~2 × 1 × 1 cm³) was sectioned and prepared for scanning (34, 48).

The micro-CT scanner has been previously described (12, 34). Myocardial samples were scanned with the use of 0.49° angular increments, providing 721 views around 360° (34). Images were recorded, digitized, and transferred to a controlling computer. The 3-D volume images, which consisted of cubic voxels of 20 µm on a side, were displayed at 40-µm on-a-side resolution (to decrease computer memory demand), and the radiopacity of each voxel was represented by a 16-bit grayscale value.

Image analysis was performed by using the Analyze software package (Biomedical Imaging Resource, Mayo Clinic, Rochester, MN) (2, 34, 48). The myocardium was three-dimensionally oriented to obtain anatomically comparable samples for study. Subsequently, the myocardium was divided into two equal parts, classified as subendocardium and subepicardium.

In each region, microvessels (diameters, <500 µm) were counted in each tomographic section with the use of object counter software and classified as either small (diameters between 80 and 200 µm) or large (diameters between 201 and 500 µm). In addition, diameters of microvessels ≥200 µm were manually measured (48). To avoid errors due to noise, microvessels <80 µm (under 2 voxels) were excluded and considered to be below resolution.

In addition, with the use of a connectivity program, a single intramyocardial artery and its branches were tomographically isolated in each pig. Their branching pattern was visually assessed in 3-D images (34, 47, 48) and compared between the groups to evaluate whether a change in microvascular density (quantified in cross-sectional images) was due to an increase in parallel vessels or due to increased branching from existing vessels.
**Myocardial tissue.** Expression of HIF-1α (the inducible subunit of HIF-1) mRNA was measured by real-time RT-PCR. Protein expression of HIF-1α and VEGF were detected by Western blot analysis (48), whereas levels of bFGF were investigated by using ELISA (Quantikine, R & D Systems, Minneapolis, MN). For localization of FGF and VEGF, immunohistochemistry was used as previously described (4, 48). Mouse monoclonal antibodies anti-VEGF (A-20, dilution 1:100, R & D Systems) and bFGF-2 (No. 147, 1:100, R & D Systems) served as primary antibodies.

**Western blot analysis.** Equal protein (50–100 μg) of myocardial homogenate was dissolved in sodium dodecyl sulfate-polyacrylamide gels (12% or 4% to 20%) under reducing conditions and electrophoretically transferred onto polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). Membranes were blocked for 1 h in Tris-buffered saline with Tween 20 (TBST)-5% nonfat milk and incubated overnight at 4°C with antibodies against HIF-1α (1:1,000, Novus Biologicals, Littleton, CO) or VEGF (1:200, Santa Cruz Biotechnology, Santa Cruz, CA), as previously described from our laboratory (3, 48). After being washed with TBST, the membranes were incubated for 1 h with horseradish peroxidase-linked anti-rabbit antibody (1:5,000, Amersham Pharmacia Biotech, Piscataway, NJ) in TBST-5% nonfat milk, and proteins were visualized by electrochemiluminescence. α-Actin (1:1,000, Sigma, St. Louis, MO) was used as the loading control (47, 48).

**Real-time RT-PCR.** Total RNA was isolated from myocardial tissue with the Trizol (Invitrogen, Carlsbad, CA) method. cDNA was synthesized with the Invitrogen Superscript First-Strand Synthesis kit, as we have previously described (47, 48).

To measure the expression of HIF-1α mRNA, real-time quantitative RT-PCR was subsequently performed with a SYBR Green jumpstart Taq ReadyMix kit (Sigma). The porcine gene-specific sequence of HIF-1α was (left) 5′-AAC AAT TCA TCT TGG CCT TC-3′ and (right) 5′-AAC AAT TCA TCT GGG CCT TC-3′. The relative amount of HIF-1α mRNA was normalized to an internal control GAPDH and relative to a calibrator (normal), calculated by 2^-ΔΔCT_. The sequence of the GAPDH primer was (upper) 5′-GTC CAT GAA CCA TGA GTG GTG-3′ and (lower) 5′-GTC TTC TGG GCA GTG AT-3′ (47, 48).

**Statistical analysis.** Values are means ± SE. Comparisons were performed by using unpaired or paired Student’s t-test (between and within groups, respectively). Statistical significance was accepted for P < 0.05. Correlation analysis was performed by least-squares linear regression using the StatView 5.1 statistical software package (SAS Institute, Cary, NC).

**RESULTS**

In hypertensive animals, MAP was significantly increased compared with MAP in normal animals, whereas PRA and LV muscle mass were not significantly different between the groups (Table 1).

**Myocardial vascular architecture.** The transmural spatial density of microvessels was significantly increased in HT animals compared with normal animals (246 ± 26 vs. 125 ± 20 vessels/cm², P = 0.02). This resulted from increased spatial density in both the subepicardium and subendocardium (Fig. 2 and Table 1) but was significantly more pronounced in the subendocardial region (Fig. 2 and Table 1). In both regions, increases in microvascular density appeared to be secondary to increased branching of small microvessels because they were tomographically connected to the main vessel (Fig. 2, bottom). The microvascular density of large microvessels was also slightly but significantly increased in both subregions (Table 1), whereas their mean diameter was significantly reduced (P < 0.01, Table 1).

**Myocardial vascular function.** Baseline BV, MBF, and MVR were not significantly different between normal and HT animals (Table 1). Matched datasets of functional responses and vessel density were available in seven HT and five control animals. The increase in BV and MBF in response to intravenous adenosine was blunted in HT animals, compared with normal animals, and was not significantly different from baseline (Fig. 3, top). MVR significantly decreased in response to adenosine in normal animals, but this decrease was blunted in HT animals (−27.7 ± 5.8 vs. −11.4 ± 11.8% compared with baseline, P = 0.02 and P = 0.16, respectively).

Interestingly, HT animals that showed the greatest increase in vessel density also showed the most attenuated BV response to adenosine. Furthermore, this relationship disclosed a significant inverse correlation between the spatial density of myocardial microvessels and the functional BV responses to adenosine. Furthermore, this relationship disclosed a significant inverse correlation between the spatial density of myocardial microvessels and the functional BV responses to adenosine in the anterior wall of HT animals (r = −0.75, P = 0.04, Fig. 3, bottom right) but not in normal animals (r = 0.01, P = 0.85, Fig. 3, bottom left).

**Ex vivo studies.** HT animals showed increased HIF-1α protein expression compared with controls (Fig. 4) with a tendency for an elevation of mRNA levels. Myocardial levels and expression of bFGF (in vascular smooth muscle cells and myocytes) and VEGF (in endothelial cells) were significantly increased in HT animals compared with normal animals and distributed uniformly throughout the myocardium (Fig. 5).

**DISCUSSION**

This study demonstrates that experimental HT induces changes in myocardial microvascular architecture and function in association with activation of growth factors, which precede the development of LVH.

HT is a major risk factor and leads to increased incidence of cardiovascular events (1, 25). HT is characterized by coronary
Fig. 2. Top: representative three-dimensional (3-D) images of transmural myocardium in normal and hypertensive (HT) pigs. Samples were scanned with micro-CT, reconstructed at resolution of 20-μm on-a-side cubic voxels and displayed at 40-μm on-a-side spatial resolution. Bottom: representative 3-D images of tomographically isolated intramyocardial coronary arteries, displayed at 20-μm on-a-side resolution, of normal and HT pigs.

Fig. 3. Top: electron beam CT (EBCT)-derived myocardial blood volume (BV, left) and myocardial blood flow (MBF, right) responses to adenosine (in % change compared with baseline) in normal (n = 5) and HT (n = 7) animals. *P < 0.05 compared with baseline. Bottom: correlation between micro-CT-measured microvascular density and EBCT-derived BV response to adenosine in anterior wall of control (left) and HT (right) animals, showing that in HT animals, but not in control, there was significant inverse correlation between microvascular structure and function.
endothelial dysfunction (42), abnormal myocardial perfusion regulation (10), and changes in myocardial microvascular architecture (9, 41). However, many of these changes were characterized in established or advanced stages of the disease, such as in the presence of LVH (9, 41). Thus it has been difficult to discern the effects of HT, per se, on the myocardium from the effects of LVH. In a model of swine renovascular hypertension, moderate increases in blood pressure for a relatively short period of time were not necessarily associated with increases in LV muscle mass (33, 35). Using this swine model of early HT, we have previously demonstrated that coronary endothelial dysfunction (35) and abnormal myocardial perfusion response to cardiac challenge (33) in early HT occur independently and before the development of LVH. The current study extends these prior observations and demonstrates that similar to microvascular dysfunction (represented in abnormal BV response to cardiac challenge), myocardial architectural changes in HT are initiated in the early stages of the disease and precede an increase in LV muscle mass, implying a direct effect of HT on both myocardial architecture and function.

To visualize and quantify the 3-D architecture of the myocardial microvasculature, we employed a unique, high-resolution micro-CT technique, which enabled detection of vessel size-specific alterations (2, 34, 48). Previous studies (34, 47) have described the capability of micro-CT to detect changes in myocardial and renal microvascular architecture in diet-induced hypercholesterolemia and ischemia. Because of its high-resolution and image analysis capabilities, this technology permits the evaluation of organ microvasculature, its distribution pattern and connectivity (34, 47). Using this technique, we observed that the HT-induced neovascularization was mainly due to an increase in the number of microvessels <200 μm in diameter, which are largely responsible for myocardial vascular resistance, and was more pronounced in the subendocardial myocardium. Indeed, the subendocardial architectural changes observed in our study likely reflect the differential sensitivity of subendocardial microvessels to nitric oxide and superoxide anion (39) or the increased propensity of this region for ischemia (28).

Fig. 4. Expression of hypoxia-inducible factor (HIF)-1α mRNA (top) and protein (bottom) in normal and HT pigs. *P < 0.05 compared with normal animals.

Fig. 5. Left: protein expression of VEGF (Western blot analysis and immunohistochemistry) in transmural myocardium of normal and HT pigs. Right: level (ELISA) and immunohistochemistry of basic FGF (bFGF) in transmural myocardium of normal and HT pigs. Myocardial expression of both bFGF and VEGF was significantly increased in HT animals. Immunohistochemistry showed that bFGF is expressed mainly in vascular smooth muscle cells and myocytes, and VEGF in endothelial cells, and that their increased expression appears to be uniform throughout myocardium. *P < 0.05 compared with normal animals.
The abnormal myocardial microvascular function seen in HT may be associated with episodes of myocardial ischemia (11) and thereby may upregulate the expression of HIF-1α (23). In addition, abnormal coronary endothelial function in early HT (35) likely contributes to tissue ischemia and increased hypoxia. These ischemic stimuli can subsequently trigger myocardial neovascularization by modulating the temporal and spatial expression and activity of a plethora of growth factors and their receptors, which may play different roles in special aspects of vessel formation (24).

VEGF and bFGF are two key angiogenic factors that have been associated with HT (16, 46) and can be induced in pathophysiological states like chronic hypoxia (15, 48). This study extends prior observations and shows that a short duration of experimental HT is sufficient to induce significant myocardial ischemia in conjunction with VEGF and bFGF activation. These data are in agreement with prior studies that have shown that hypoxia serves as a powerful stimulus for the activation of VEGF (48) and bFGF (17, 43). In addition, myocardial neovascularization observed in our study could have also resulted from the synergistic interaction of VEGF and bFGF with alternative pathways, such as the local renin-angiotensin system (29) or other angiogenic factors.

In addition, we observed that HT was associated with a decrease in mean diameter of large microvessels, possibly due to inward growth of the vascular wall (negative vascular remodeling). Both the expression of angiogenic factors (16) and sensitivity to their growth stimulatory actions (6) may be enhanced in HT, which may contribute to vascular wall thickening (33). The increased number of small microvessels in the face of the decreased diameter of large microvessels might have been responsible for maintaining an unaltered basal microvascular blood volume and MBF, as previously observed in asymptomatic patients with borderline hypertension and no LVH (19). A slight, albeit not significant, increase in MBF that paralleled the increase in MAP likely sustained MVR.

The powerful combination of anatomical assessment via micro-CT with functional assessment of myocardial vascular function using EBCT allowed exploration of the relation between microvascular anatomy and function, which becomes critically important in view of the growing interest in therapeutic interventions targeted to increase the number of vessels (38) with the ultimate goal of improving organ function. Interestingly, we observed that microvascular neovascularization was inversely related to myocardial BV response to challenge in early HT, implying that an increase in the number of vessels did not translate into an improvement of myocardial function but, on the contrary, was associated with a worsening of the myocardial functional response. This may be due to abnormal features like disorganized architecture (8) and functional abnormalities (7) that can characterize newly generated microvessels and that may lead to altered regulation of flow to the myocardium. Nevertheless, because pathological neovascularization (as in HT) may have different mechanisms and effects than therapeutic neovascularization, these results should not be extrapolated to states where therapeutic angiogenesis might be beneficial.

Limitations. In the current study, we describe the microvascular changes that occur in early hypertension, mostly in vessels <200 μm in diameter. Although the resolution of micro-CT is as high as 4-μm voxels, limited computing power currently restricts routine handling of such large image files. In the current study, we used 40-μm voxels, and an enhancement of less than two adjacent voxels was considered as noise so that analysis was restricted to myocardial microvessels ≥80 μm. Rapid progress in memory size and computing power will likely allow future analysis of much smaller vessels.

It is also possible that microvessels might have been misclassified due to vascular remodeling or residual vascular tone with a consequent decline in the diameter of large microvessels. However, this is unlikely because our tissue preparation allows for full vascular relaxation, and the number of large microvessels was, in fact, increased rather than decreased.

In this study we used relatively young pigs, which may show greater potential for angiogenic responses compared with older animals (30). Notably, we also used female pigs, and estrogen has been reported to increase angiogenic response in HT (40). However, the pigs in our experimental groups were premenstrual, so that hormonal changes were unlikely to interfere with our results. Nevertheless, the possibility of effects from age and sex differences cannot be ruled out.

In summary, this study demonstrates that myocardial microvascular changes in early HT are associated with hypoxia and growth factor activation, as well as with microvascular functional changes. Importantly, we have shown that architecture changes in the myocardial microcirculation precede development of LVH and that an increase in its density did not translate into improved myocardial microvascular function in HT. These observations support the notion that early hypertension has direct functional and structural effects on the myocardial microcirculation. These findings have clinical implications because these changes occur after a relatively short period of moderate HT. These results underscore the functional significance of the architectural changes present in early HT and support early application of intensive preventive and therapeutic strategies in patients with HT.

GRANTS

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REFERENCES


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