Role of Oxidative Stress in Remodeling of the Myocardial Microcirculation in Hypertension

Xiang-Yang Zhu, Elena Daghini, Alejandro R. Chade, Martin Rodriguez-Porcel, Claudio Napoli, Amir Lerman, Lilach O. Lerman

Objective—We tested the hypothesis that in early hypertension (HT), increased oxidative stress leads to myocardial microvascular remodeling.

Methods and Results—Pigs were studied after a 12-week observation: normal (n=8), untreated renovascular HT (n=8), or HT + chronic antioxidant supplementation (HT + A, n=6). Left ventricular muscle mass (LVMM) and myocardial blood flow (MBF) reserve were determined using electron beam computer tomography (CT), and the spatial density and tortuosity of myocardial microvessels (<500 μm) was then measured in myocardial samples with micro-CT. Myocardial microvascular morphology, oxidative stress, inflammation, and growth factor expression were determined in vitro. HT and HT + A had similarly increased arterial pressure and LVMM, but only HT showed impaired MBF response to adenosine. Compared with normal, HT had increased spatial density of myocardial microvessels, which was preserved in HT + A (111.8 ± 7.8, 166.3 ± 15.7, and 106.4 ± 6.1 vessels per cm², respectively). HT also showed microvascular wall thickening, increased systemic and tissue oxidative stress, inflammation, and expression of vascular endothelial growth factor and its receptor Flik-1, most of which were attenuated by antioxidants.

Conclusions—Myocardial microvascular remodeling in early HT is accompanied by tissue oxidative stress, inflammation, and altered growth factor expression, and attenuated by antioxidant intervention. This study underscores a role of increased oxidative stress in modulating myocardial microvascular architecture in early HT. (Arterioscler Thromb Vasc Biol. 2006;26:1746-1752.)

Key Words: microcirculation ■ atherosclerosis ■ hypertension ■ oxidative stress ■ inflammation

Hypertension (HT) is a major risk factor for atherosclerosis and ischemic heart disease. We have previously demonstrated that early experimental HT was associated with impaired myocardial perfusion, partly mediated by increased oxidative stress, which was corrected with chronic antioxidant supplementation. However, the potential effect of antioxidants on coronary microvascular remodeling in early HT remains unclear.

Reactive oxygen species (ROS) may directly upregulate the expression of vascular endothelial growth factor (VEGF), a potent angiogenic growth factor that stimulates endothelial cell proliferation and migration in vitro and angiogenesis in vivo. Recruitment of inflammatory cells and their interaction with vascular endothelial cells may also enhance angiogenesis. Furthermore, ROS derived from NAD(P)H oxidase, such as superoxide anion, also upregulate the expression of inflammatory factors such as monocyte chemoattractant protein (MCP-1) or tumor necrosis factor (TNF-α), which in turn enhance angiogenesis by upregulating VEGF expression. The angiogenic effects of VEGF are mediated largely by its receptor Flik-1 and facilitated by cell surface receptors like alpha(v)beta3 integrins, which are expressed on angiogenic vessels. However, despite the redox sensitivity of VEGF expression, and the ability to improve microvascular architecture in other disease states like hypercholesterolemia, the efficacy of chronic antioxidant intervention to attenuate myocardial microvascular remodeling in HT has not been fully explored.

Micro-computed tomography (μ-CT) is a powerful imaging technique that permits assessment of the 3D pattern of the microvascular structure in situ. We have previously demonstrated the feasibility of studying with μ-CT the 3D myocardial microvascular architecture and tortuosity, a unique marker that characterizes angiogenic vessels.

Therefore, this study was designed to test the hypotheses that in early HT coronary microvascular remodeling is driven by oxidative stress and would be attenuated during chronic antioxidant supplementation.
Methods

All procedures using animals were approved by the Institutional Animal Care and Use Committee. Three groups of female domestic pigs (50 to 60 kg; Larson Products, Sargeant, Minn.) were studied after 12 weeks of no intervention (normal, n=8), renovascular HT (n=8), or HT+dietary antioxidant supplementation (100 IU/kg of vitamin E and 1 g of vitamin C daily, n=6). HT was achieved by placement of a local-irritant coil in the main renal artery at baseline, which induced gradual development of unilateral renal artery stenosis and subsequent development of HT, as previously described. Antioxidant intervention commenced 1 day after this procedure. We have previously shown that this antioxidant combination was effective in decreasing oxidative stress and normalizing vascular function. Mean arterial pressure (MAP) was measured in all animals by a PhysioTel telemetry system (Data Sciences) implanted at baseline in the left femoral artery.10

After 12 weeks of observation, the animals were anesthetized (ketamine 17.5 mg/kg and xylazine 2.3 mg/kg/h in saline), and catheters were placed in the right atrium for electron beam CT (EBCT) scanning. Blood samples were collected during catheter placement, and plasma TNF-α level (R&D Systems) and total superoxide dismutase (SOD) activity were determined, following manufacturer’s instruction.

EBCT Scanning

To evaluate myocardial flow reserve and left ventricular muscle mass (LVMM), animals were scanned by the EBCT (Imatron C-150), as previously described. Initially, 40 consecutive ECG-triggered end-diastolic scans were acquired at 1 to 3 heartbeat intervals after a bolus injection of the contrast medium iopamidol (0.5 mL/kg over 2 seconds). After a 15-minute recovery, the flow study was repeated toward the end of a 15-minute infusion of adenosine (400 µg/kg/min). Blood pressure and heart rate were recorded before and after adenosine infusion. Another 15 minutes later, 8 end-diastolic scans from LV apex to base were acquired simultaneously for determination of LVMM.

Images were analyzed by tracing regions of interest in the LV cavity and the anterior wall. In addition, a subendocardial region of interest was defined in the inner half of the anterior wall. The changes in density over time in each region were subsequently plotted as time-attenuation curves that were fitted by an extended γ-variate model. The area and first moment of each curve were then used for calculation of intramyocardial blood volume (BV) and mean transit time (MTT). Myocardial perfusion (ml/min/g) was calculated as: 60×(BV/MTT)/(1.05×(1–BV)). LVMM was calculated from the LV myocardial area, which was obtained by manually tracing the endocardial and epicardial borders of the LV at each of the 8 levels, and multiplied by slice thickness. Myocardial blood flow (MBF) was subsequently calculated as myocardial perfusion/LVMM.

After euthanasia, performed by intravenous pentobarbital sodium (100 mg/kg, Sleepaway), the heart was removed for in vitro studies. In addition, 1 to 3 intra-myocardial arterioles and their branches were tomographically isolated in each pig, and vessel tortuosity (elongation factor) was determined by dividing the 3D path distance (total length) by the linear distance (shortest distance between end points) of the main branches.

Myocardial Tissue

Myocardial tissue oxidative stress was assessed by in situ production of superoxide anion (using the oxidative fluorescent dye Dihydroethidium [DHE]), and by protein expression of NAD(P)H oxidase (subunits p47phox and p67phox) and nitrotyrosine, the footprint of peroxynitrite. Myocardial inflammation was evaluated by protein expression of MCP-1, TNF-α, as well as myeloperoxidase (MPO), an enzyme derived from infiltrating inflammatory cells. Myocardial fibrosis and microvascular morphology were evaluated by trichrome and α-smooth muscle actin, and growth factor activity by protein expression of VEGF, Flk-1, and by alpha(γ)beta3 integrin immunostaining.

Western Blotting

Myocardial homogenate (100 µg of protein) was dissolved in SDS-polyacrylamide gels (10% or 15%) following standard Western blotting protocol. Membranes were incubated with antibodies against p47phox, p67phox, VEGF, TNF-α (1:200 Santa Cruz Biotechnology), Flk-1 (1:200, NeoMarkers), MCP-1 (1:500, BioVision), and MPO (1:500, US Biological) at 4°C overnight, and horseradish peroxidase–linked anti-rabbit or anti-mouse antibody (1:5000, Amersham Pharmacia Biotech) was used as secondary antibody. β-actin (1:1000, Sigma) was used as the loading control.

DHE Staining

In situ production of superoxide anion was assessed in 30-µm frozen myocardial sections using the oxidative fluorescent dye DHE and evaluated under fluorescence microscopy (Texas red light), as we have previously described.13

Histology

Fixed blocks of tissue were embedded in paraffin, and 5-µm-thick sections were cut from each block and stained with trichrome or elastin van Gieson and 5-µm-thick sections were cut from each block and stained with trichrome or elastin van Gieson and eosin (Epon) and examined, and microvascular media/lumen ratio measured using standard techniques.

Statistical Analysis

Continuous data are expressed as mean±SEM. Multiple group comparisons used analysis-of-variance, followed by t test with the Bonferroni correction, when applicable. Statistical significance was accepted if P≤0.05. Protein expression was assessed relative to the loading control (actin) and expressed as ratio.

Results

Myocardial Microvascular Function

MAP and LVMM were increased similarly in both the HT and HT+antioxidants groups, indicating that antioxidant vitamins had no effects on blood pressure and LV hypertrophy (LVH) (Table 1). Blood pressure and heart rate showed a transient decrease and increase, respectively, but stabilized after a few minutes of adenosine infusion, and by the time of EBCT scanning (15 minutes after initiation of infusion), were similar to pre-adenosine values (Table 1). There were no significant differences in baseline MBF and ejection fraction among the 3 groups. However, MBF response to adenosine was significantly attenuated in HT pigs, suggesting impaired...
coronary flow reserve, which was significantly improved by antioxidants (Table 1). Baseline subendocardial perfusion was increased in HT, but its response to adenosine was impaired, suggesting impaired subendocardial coronary flow reserve, which was improved by antioxidants (Table 1).

### Microvascular Remodeling

The spatial density of microvessels (<500 μm) assessed by micro-CT was significantly higher in HT than in normal (166.3±15.7 versus 111.8±7.8 vessels per cm², P<0.05), an increase that was more pronounced in the subendocardium and appeared to result from increased microvascular sprouting from intramyocardial arteries (Table 2; Figure 1). Neovascularization was observed in HT pigs only in subendocardial vessels smaller than 300 μm, and was normalized in HT+antioxidants (106.4±6.1 vessels per cm²). In addition, HT had increased vascular volume fraction and microvascular tortuosity (Figure 1; Table 2), while tomographic microvascular diameter was not significantly changed. All of the tomographic signs of microvascular remodeling were significantly improved by antioxidant supplementation.

The micro-CT findings were corroborated by increased positive staining of integrins in HT (supplemental Figure I, available online at http://atvb.ahajournals.org). HT pigs also showed increased expression of VEGF and its recep-

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| TABLE 1. Systemic Characteristics and Myocardial Microvascular Function in Normal, Hypertension (HT), and HT+Antioxidants Pigs |
|---------------------------|----------------|----------------|
|                          | Normal          | HT             | HT+Antioxidants     |
| Basal mean arterial pressure, mm Hg | 110.5±4.6       | 131.3±9.8*     | 132.1±5.4*          |
| Change during adenosine infusion, % | −8.7±2.1       | −11.8±4.6      | −8.0±2.6            |
| Basal heart rate, beats/min | 81±7            | 68±3           | 83±8                |
| Change during adenosine infusion, % | +2.7±6.4       | +9.3±5         | +0.2±3.2            |
| Plasma rennin activity, ng/ml/hr | 0.33±0.1        | 0.27±0.05      | 0.33±0.03           |
| Tumor Necrosis Factor alpha, pg/ml | 65.5±4.5       | 122.1±32.9*    | 126.8±25.2*         |
| SOD activity, U/ml | 95.5±13.2       | 53.8±11.1*†    | 91.2±6.3            |
| Left Ventricular Muscle Mass, g/kg BW | 2.2±0.1         | 2.6±0.1*       | 2.6±0.2*            |
| Ejection fraction, % | 49±2.4          | 51.1±3.1       | 47.3±1.7            |

### Myocardial blood flow

|                          | Normal          | HT             | HT+Antioxidants     |
| Baseline | 100.2±5.7       | 118.7±12.9     | 106.4±13.7          |
| Adenosine | 177.5±13.2#    | 136±11.6*†    | 242.9±80#           |

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*P<0.05 vs Normal, †P<0.05 vs HT+antioxidants, #P<0.05 vs baseline.

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| TABLE 2. Structural Characteristics of Myocardial Microvessels in Normal, Hypertension (HT), and HT+Antioxidants Pigs |
|---------------------------|----------------|----------------|
|                          | Normal          | HT             | HT+Antioxidants     |
| n | 8 | 8 | 6 |
| Spatial density (vessels/cm²), subendocardial |
| ≤200 μm | 108±7            | 191±23*†       | 119±11              |
| 201–300 μm | 9±3             | 18±2*†         | 8±1                 |
| 301–500 μm | 3±1            | 4±2            | 5±1                 |
| Subepicardial |
| ≤200 μm | 91±16           | 107±12         | 76±5                |
| 201–300 μm | 9±2            | 10±2           | 4±1                 |
| 301–500 μm | 3±1           | 3±1            | 2±0                 |
| Tortuosity, dimensionless | 1.02±0.01      | 1.31±0.03*†   | 1.22±0.01*          |
| Average Diameter, μm | 147.1±9.8      | 140.5±6.4     | 156.4±6.9           |
| Vascular media/lumen ratio | 0.43±0.03      | 1.1±0.22*†    | 0.58±0.12           |
| Dihydroethidium, % of surface area | 1.5±0.1        | 4.3±0.3*†    | 2.8±0.2*            |

*P<0.05 vs Normal, †P<0.05 vs HT+antioxidants.
tor Flk-1 (Figure 2), indicating angiogenic activity. Moreover, trichrome staining showed increased perivascular fibrosis compared with normal, and HT also exhibited greater α-smooth muscle actin immunoreactivity (supplemental Figure I) and vascular media-to-lumen ratio (Table 2), which were attenuated by antioxidant supplementation.

**Oxidative Stress**

Increased myocardial expression of p47phox and p67phox NAD(P)H oxidase (Figure 2) and increased DHE (supplemental Figure I; Table 2) fluorescence throughout the myocardial sample in HT indicated increased superoxide production. Indeed, nitrotyrosine expression was increased in the HT myocardium, especially in endothelial cells and myocytes of the subendocardial region (supplemental Figure II), while eNOS expression was similar among the 3 groups (Figure 2). In addition, systemic SOD activity was decreased in HT (Table 1), suggesting blunted radical scavenging activity. All these parameters were preserved in HT animals chronically supplemented with antioxidants.

**Inflammation**

HT increased protein expression of MCP-1, which was located mainly in endothelial cells, smooth muscle cells, and perivascular inflammatory cells, suggesting inflammatory cells recruitment, which was further supported by increased myocardial expression of MPO (Figure 2; supplemental Figure II). Systemic level of TNF-α was similarly increased in both HT and HT + antioxidants animals compared with normal (Table 1), indicating that antioxidants had no effect on this systemic inflammatory marker. On the other hand, increased myocardial expression of TNF-α in HT was significantly attenuated by antioxidants (Figure 2), suggesting a more pronounced antiinflammatory effect of the vitamins at the tissue level.

**Discussion**

This study demonstrates that myocardial microvascular remodeling in early HT is accompanied by increased oxidative stress, inflammation, and VEGF expression, and attenuated by antioxidant intervention. This study supports a role for endogenous oxidative stress in myocardial microvascular remodeling in HT.

HT is an important risk factor for development and progression of cardiovascular diseases. We have previously shown that early HT is associated with increased oxidative stress, a state of excessive abundance of ROS, as well as microvascular dysfunction, which was improved by long term antioxidant intervention. This study extends those findings, and demonstrates that early HT also induced myocardial...
microvascular remodeling, which was normalized by antioxidant intervention. Microvascular remodeling was characterized by increased vessel density and tortuosity, a feature commonly observed in newly formed vessels. Increased myocardial microvascular density was also corroborated by integrin $\alpha_3\beta_3$ immunohistochemistry. Integrins are a family of adhesion molecules that participate in a wide range of biological processes, including angiogenesis. Integrin $\alpha_3\beta_3$ is an endothelial coreceptor for VEGF, which is expressed on proliferating endothelium of angiogenic vessels. Our findings suggest that its expression is redox sensitive, as shown for other members of the integrin family.\(^{15}\)

Increased oxidative stress in myocardial tissue was likely at least partly responsible for the observed neovascularization. Although increased DHE fluorescence may be caused by cellular trauma, the significant difference among the groups, and the increased expression of NAD(P)H oxidase, support the notion of increased oxidative stress in our HT model. The superoxide anion is an important ROS, which is mainly generated by NAD(P)H oxidase.\(^{16}\) In the current study, HT was found to increase the in situ generation of superoxide in the myocardium, likely consequent to increased expression of NAD(P)H oxidase, represented by p47phox and p67phox, which are needed for enzyme assembly and activity. The superoxide anion may have several effects on microvascular remodeling, either directly or through the generation of other radicals. ROS upregulate VEGF expression in human vascular smooth muscle cells\(^{17}\) and are increasingly recognized as direct signaling mediators both upstream and downstream\(^{18}\) to VEGF. Therefore, increased oxidative stress may modulate neovascularization via several different signaling pathways,\(^{19}\) among which increased VEGF expression is particularly potent.\(^{20}\) Accordingly, this study shows that both VEGF and its receptor Flk-1 were increased in HT and normalized by antioxidant intervention. Flk-1 is the major mediator of the mitogenic, angiogenic, and permeability-enhancing effects of VEGF.\(^{21}\) Furthermore, chronic blockade of oxidative stress not only preserved the expression of VEGF, but also normalized myocardial microvascular architecture in experimental HT, suggesting an association between oxidative stress and myocardial microvascular remodeling, which may be mediated via VEGF.

Antioxidants may attenuate this cascade at multiple points, and their beneficial effect may also be attributable to decreased inflammation. Notably, this study shows in HT increased expression of both MCP-1 and MPO, which are important inflammatory factors. The enzyme MPO transforms low-density lipoprotein into atherogenic particles, interacts with vascular NAD(P)H oxidase, and is involved in exacerbation of vascular diseases under inflammatory conditions.\(^{22}\) MCP-1 is the major regulator of macrophage recruitment to vessels and plays an important role in inflammation. Indeed, superoxide derived from NAD(P)H oxidase mediates TNF-$\alpha$-induced MCP-1 expression in endothelial cells.\(^{5}\) Furthermore, MCP-1 can induce angiogenesis by induction of VEGF-A gene expression\(^{23}\) and may participate in instigation of myocardial fibrosis by upregulating transforming growth factor (TGF)-$\beta$ expression and fibroblast proliferation to induce perivascular fibrosis,\(^{24}\) as observed in this study. Interestingly, we found that antioxidants had no effect on systemic TNF-$\alpha$ levels but attenuated its myocardial expression, suggesting that the antiinflammatory effect of the vitamins was at the tissue level. Nevertheless, local inflammation status may be more important than systemic for myocardial microvascular remodeling in HT. Antioxidants attenuated MCP-1 expression and perivascular myocardial fibrosis, indicating involvement of oxidative stress in this mechanism.

Altersations in vascular density have been suggested to be the most efficient mechanism for long-term regulation of the microcirculation.\(^{25}\) Furthermore, the pronounced neovascularization and increased expression of nitrotyrosine in the
subendocardium are in agreement with the greater oxygen consumption in this region compared with the subepicardium, and thus relative vulnerability to ischemia. In line with the greater microvascular density, we observed in HT increased basal subendocardial myocardial perfusion but impaired response to adenosine, as previously observed. The increased BV and MBF during IV adenosine infusion in our model was elicited by increased myocardial demand and involved both endothelium-dependent and -independent mechanisms. The mechanism by which MBF reserve was attenuated in HT despite an increase in capillary density may be speculated. It is likely caused by an altered structure or endothelial function, these newly formed vessels do not function normally and cannot vasodilate in response to increased myocardial demand, as recently shown. This hypothesis is supported by our observation that a decrease in the proportion of these dysfunctional vessels in HT+antioxidants improved overall microvascular response to challenge. For example, decreased bioactivity of nitric oxide by its interaction with superoxide to form nitryosyline likely impaired MBF reserve in HT, because we observed increased superoxide and nitryosyline whereas eNOS expression was unaltered. The process of vascular remodeling, as reflected in the increase in media/lumen ratio, may also contribute to the attenuated vasorelaxation of these vessels. Furthermore, myocardial microvessels also showed in HT increased tortuosity, which characterizes angiogenic vessels, increases vascular resistance, and may diminish myocardial perfusion reserve. Although not fully normalized, the decreased tortuosity by antioxidants in our study suggests a role for oxidative stress in this mechanism. Microvascular rarefaction has been described in HT, but most of these studies typically measured capillary density in the peripheral microcirculation using histological techniques. In the current study, we used a high resolution micro-CT to assess the 3D pattern of the microvascular structure in situ, including their spatial distribution. We observed that arteriolar density was increased in early HT, consistent with previous studies showing increased arteriolar or capillary density in rat and mouse HT models. A recent study showed that coronary angiogenesis was initially enhanced during adaptive cardiac growth, in association with induction of myocardial VEGF, but subsequently reduced as hearts underwent pathological remodeling. Indeed, in our early HT model, LVH was likely evolving, and microvascular density still exceeded the increase in LVM. In chronic and prolonged HT, inadequate growth (or loss) of the coronary microvascular bed relative to the increase in LVM at that stage may result in a decrease in microvascular density and further impair myocardial perfusion. Microvascular remodeling in HT was also reflected in their thickening. Media to lumen ratio of subcutaneous small arteries was shown to be greater in hypertensive patients and significantly associated with the occurrence of cardiovascular events. In the current study, histological observation showed increased media/lumen ratio, whereas average diameter assessed by micro CT remained unchanged. It is possible that thickening of the vascular wall precedes a decrease in luminal diameter, as shown in a rat HT model. Its improvement in our study may suggest that antioxidants might be beneficial for the outcomes of hypertension. However, future studies will be needed to determine their ability to reverse, rather than prevent microvascular remodeling.

Both vitamin E and vitamin C are important dietary antioxidants. Vitamin C regenerates vitamin E, and vitamin E radicals constitute an additional substrate for vitamin C, resulting in synergistic antioxidant effect. In clinical practice, self-selected supplementation of vitamin E has been associated with reduced coronary events and atherosclerotic progression, but the evidence from clinical trials is controversial. The recent Antioxidant Supplementation in Atherosclerosis Prevention (ASAP) study suggested that supplementation with combination of vitamin E and slow-release vitamin C slows down atherosclerotic progression in hypercholesterolemic persons. However, other randomized trials had shown no significant effects of antioxidant vitamin supplementation on cardiovascular endpoints. Differences in study population, the duration, dose, and type of supplements, as well as outcome measures may explain this variability. For example, in clinical studies basal oxidative stress often has not been assessed, and some patient groups may not have had increased oxidative stress, while in other groups secondary rather than primary prevention has been attempted. Thus, antioxidants may speculatively confer beneficial effects only when basal pre-existing oxidative stress is increased, like in hypertension and hypercholesterolemia.

In summary, we observed that altered myocardial microvascular architecture in HT was accompanied by increased oxidative stress and inflammation and was preserved by chronic antioxidant intervention. Therefore, our study suggests a role for increased oxidative stress in myocardial microvascular remodeling in HT.

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Disclosure(s)
None.

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Figure legends

Figure I. Top: Increased superoxide presence in myocardial tissue of HT, as indicated by increased red fluorescence in DHE staining. Middle: \( \alpha \)-smooth muscle actin (\( \alpha \)-SMA) staining, showing increased wall thickness of microvessels in HT (arrow). Bottom: Integrin beta3 staining, showing positive immunoreactivity (red) on an angiogenic vessel in HT (arrow).

Figure II, Top: Immunohistochemistry of MCP-1 shows staining mainly in endothelial cells, smooth muscle cells, and perivascular inflammatory cells in HT. Middle: Expression of nitrotyrosine (red) in HT was evident mainly in endothelial cells and myocytes of subendomyocardium (x40). Bottom: Trichrome staining (blue) showing perivascular fibrosis in HT (x20).
Figure I
Figure II

MCP-1

Nitrotyrosine

Trichrome

Normal | HT | HT+antioxidants