Long-Term Antioxidant Intervention Improves Myocardial Microvascular Function in Experimental Hypertension

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Abstract—Hypertension increases oxidative stress, which can impair myocardial microvascular function and integrity. However, it is yet unclear whether long-term antioxidant intervention in early hypertension would preserve myocardial perfusion and vascular permeability responses to challenge. Pigs were studied after 12 weeks of renovascular hypertension without (n=8) or with daily supplementation of antioxidants (100 IU/kg vitamin E and 1 g vitamin C, n=6), and compared with normal controls (n=7). Myocardial perfusion and microvascular permeability were measured in vivo by electron beam computed tomography before and after 2 cardiac challenges (intravenous adenosine and dobutamine). Basal left ventricular muscle mass was also obtained. Mean arterial pressure was significantly increased in both groups of hypertensive animals (without and with antioxidants, 123±9 and 126±4 mm Hg, respectively, versus normal, 101±4 mm Hg; both P<0.05), but muscle mass was not different among the groups. The impaired myocardial perfusion response to adenosine observed in hypertensives (normal, +51±14%; P<0.05 versus baseline; hypertension, +14±15%; P=0.3 versus baseline) was preserved in hypertensive pigs that received antioxidants (+44±15%; P=0.01 compared with baseline). Long-term antioxidant intervention also preserved subendocardial microvascular permeability responses in hypertension. On the other hand, antioxidant intervention had little effect on the hypertension-induced myocardial vascular dysfunction observed in response to dobutamine. This study demonstrates that the impaired myocardial perfusion and permeability responses to increased cardiac demand in early hypertension are significantly improved by long-term antioxidant intervention. These results support the involvement of oxidative stress in myocardial vascular dysfunction in hypertension and suggest a role for antioxidant strategies to preserve the myocardial microvasculature. (Hypertension. 2004;43[part 2]:493-498.)

Key Words: antioxidants • hypertension, experimental • imaging • oxidative stress

Hypertension (HT) is associated with impaired coronary endothelial function, both in vivo1 and in vitro,2 and can impair myocardial perfusion,3 especially after development of left ventricular hypertrophy (LVH).4-5 In addition to its effect on vascular reactivity, HT has effects on other functions of the vascular wall. Increases in blood pressure can also lead to alterations in thrombogenesis6 and increased permeability of the endothelium.7,8 One of the mechanisms that might be responsible for HT-induced myocardial dysfunction is an increase in oxidative stress, which has been demonstrated in both humans9 and animal models.10 Indeed, short-term antioxidant administration improved coronary epicardial endothelial function.9,11 However, whether long-term antioxidant supplementation might preserve HT-induced myocardial microvascular dysfunction remains unclear.

Electron beam computed tomography (EBCT), a fast CT scanner, provides a unique tool to accurately12 and reproducibly13 study in vivo transmurral myocardial perfusion14-16 and myocardial microvascular permeability (MVP)15-17 noninvasively. This tool provides an opportunity to explore noninvasively the effect of long-term therapeutic interventions on myocardial vascular function. Thus, the present study was designed to test the hypothesis that long-term antioxidant blockade would preserve myocardial vascular function in early HT.

Methods

Animals

All of the study procedures were approved by the Institutional Animal Care and Use Committee. Twenty-one female, domestic crossbred pigs (Pork Partners, Stewartville, Minn; 55 to 65 kg each) were studied after a 12-week preparation: one group (normal, n=7) was untreated, and in the second (HT, n=8), renovascular HT was induced by placing a local-irritant stent in the left renal artery, as previously described.16,18 In addition to induction of renal artery stenosis, animals from group 3 (HT+antioxidants [Ao], n=6) received daily oral antioxidant supplementation (combination of 100 IU/kg vitamin E and 1 g vitamin C). This dosage was based on our previous studies in pigs that showed that this combination provided...
an effective blockade of the endogenous oxidative stress cascade.\textsuperscript{15,19,20}

After 12 weeks of observation, blood samples were collected for measurement of plasma renin activity (by radioimmunoassay), superoxide dismutase (SOD) activity, and plasma vitamin E and C levels (by high-performance liquid chromatography).\textsuperscript{15,16} EBC in vivo studies were then performed to assess myocardial perfusion, MVP, cardiac hemodynamics, and LV muscle mass. Myocardial functional studies were performed under rest conditions and repeated after cardiac challenge with intravenous adenosine and intravenous dobutamine, substances routinely used for cardiac stress testing.

**EBCT Scans**

Animals were anesthetized with ketamine and xylazine (20 mg/kg and 2 mg/kg, respectively), intubated, and ventilated. Anesthesia was maintained with a constant infusion of ketamine (17.5 mg/kg per hour) and xylazine (2.5 mg/kg per hour). Catheters were placed fluoroscopically in the aorta for measurement of mean arterial pressure (MAP) and in the right atrium (for contrast media injections and drug infusions).\textsuperscript{15,16} Animals were then positioned in the EBCT (C-150, Imatron Inc), and blood samples were collected. Two mid-LV levels were identified, and a baseline myocardial functional (perfusion and MVP) study was performed. Forty consecutive end-diastolic scans were obtained during 40 seconds (at 1- to 3-beat intervals) after a 2-second injection of iopamidol-370 (0.3 mL/kg, Squibb) into the right atrium.\textsuperscript{15,16,21} Myocardial functional studies were repeated at 20- to 30-minute intervals after intravenous infusion of either adenosine (400 µg/kg per minute) or dobutamine (15 µg/kg per minute, to a target heart rate of 150 bpm). This was followed 15 minutes later by a myocardial volume study, as previously described.\textsuperscript{14} After completion of the in vivo studies, animals were killed by intravenous pentobarbital sodium (100 mg/kg, Fort Dodge Laboratories).

**EBCT Data Analysis**

For the calculation of LV ejection fraction, the endocardial borders were traced at end-diastole and end-systole on the volume study, and the LV ejection fraction and stroke volume were calculated as previously described.\textsuperscript{21} Cardiac output was then calculated as stroke volume×heart rate, and systemic vascular resistance (SVR) was calculated as 80×MAP/cardiac output. For measurement of LV muscle mass, the epicardial and endocardial LV surfaces were traced at end-diastole, and the product of myocardial muscle area, myocardial specific density (1.05 g/mL), and slice thickness was calculated.\textsuperscript{16,21}

For measurement of myocardial vascular function, regions of interest were traced in the anterior cardiac wall and LV chamber.\textsuperscript{14} For transmural distribution, the myocardial region of interest was further subdivided into equidistant subepicardium and subendocardium. Myocardial perfusion (mL/min per gram) was calculated from tissue time-density curves by using the ratio of intravascular volume fraction and mean transit time, according to previously validated algorithms.\textsuperscript{14,15,16} Perfusion of the subendocardial and subepicardial regions was similarly obtained and their ratio (endocardial/epicardial) calculated. Myocardial blood flow was subsequently calculated as perfusion×LV muscle mass. Then, myocardial vascular resistance (MVR) was calculated as 80×MAP/myocardial blood flow.\textsuperscript{16,22} MVP (arbitrary units [AU]) was calculated as 60×1.05×[slope of extravascular curve×mean transit time]/area under the input curve/blood volume.\textsuperscript{15,16} where slope is the maximal slope of the ascending arm of the extravascular curve. Blood volume was used as a surrogate for vascular surface area.\textsuperscript{17} MVP of the subendocardial and subepicardial regions at baseline and in response to challenge was similarly obtained.

**Scavenging Activity**

Total SOD activity was measured in plasma with a commercially available kit (Cayman Chemical superoxide dismutase assay kit) according to the vendor’s instructions. In brief, blood anticoagulated with EDTA was centrifuged twice at 4°C and the supernatant collected. The standards and samples were placed in a sample plate and assayed in duplicate. Reaction was initiated by adding 20 µL diluted xanthine oxidase to all wells, and then the plate was incubated on a shaker at room temperature for 20 minutes. The absorbance of each standard and samples was read at 450 nm with a plate reader. SOD activity was calculated from the linear regression of the standard curve after subtracting the linearized rate for each sample. One unit was defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. To inhibit the CuZn isoform of SOD, 1 to 3 mmol/L KCN was added to the assay, thereby disclosing the activity of Mn-SOD alone. The activity of CuZn-SOD was then calculated as total SOD−Mn-SOD activity.

**Statistical Analysis**

Results are expressed as mean±SEM. Comparisons within (adenosine/dobutamine versus baseline) and between (normal versus HT, HT versus HT+Ao) groups were performed by ANOVA, unpaired Student t test (by itself or as a post hoc test), or paired Student t test when applicable. Statistical significance was accepted for a value of P<0.05.

**Results**

**General Group Characteristics**

Untreated HT animals had a significant increase in MAP compared with normal controls, whereas basal heart rate did not differ significantly among the groups (Table). After 12 weeks of daily antioxidant supplementation, the HT+Ao group had increased levels of vitamins C and E compared with normal and HT pigs (Table) and MAP similar to that of untreated HT animals (Table). There was no difference in plasma renin activity, LV ejection fraction, and muscle mass among the groups (Table). Untreated HT pigs had significantly decreased basal stroke volume and cardiac output and increased SVR, which were slightly but not significantly improved in the HT+Ao group (Table). Changes of MAP in response to intravenous adenosine were similar among the groups (normal, −4±4%; HT, −7±5%; and HT+Ao, −9±2% compared with baseline; ANOVA P=0.43), whereas heart rate remained unchanged (data not shown). Infusion of intravenous dobutamine induced an expected increase in heart rate (by protocol design; data not shown) but no change in MAP (at the time of the functional study).

Early HT induced a significant decrease in the activity of the endogenous radical scavenger enzyme system SOD (Table), evident in both the CuZn-SOD and Mn-SOD isoforms (Table). Long-term antioxidant supplementation preserved endogenous scavenging activity, as evidenced by normalization of the activity of total SOD and its 2 isoforms (Table).

**Myocardial Perfusion**

Baseline anterior wall myocardial perfusion and MVP were similar among the 3 groups (Table), as was the endocardial-epicardial perfusion ratio (normal, 1.00±0.13; HT, 1.11±0.07; and HT+Ao, 1.11±0.10; ANOVA, P=0.7). Adenosine infusion induced a significant increase in anterior wall myocardial perfusion in normal pigs (to 1.24±0.17 mL/min per g tissue; P<0.01; Figure 1A) that was accompanied by a significant decrease in MVP (Figure 1B). The increase in myocardial perfusion was observed in the both the subepicardium and subendocardium but tended to be more pronounced in the subendocardium (endocardial-epicardial
perfusion ratio rose to 1.15±0.14; \( P=0.06 \). On the other hand, the myocardial perfusion response of HT pigs to intravenous adenosine was blunted (to 1.12±0.22 mL/min per g tissue; \( P=0.3 \); Figure 1A), as was the MVR response (Figure 1B); this impairment was spatially homogenous, because there was no change in subepicardial or subendocar-
swine renovascular HT can be improved by long-term antioxidant intervention. HT has been associated with alterations in myocardial vascular function, especially after development of LVH. Furthermore, we have recently shown that early experimental HT alters myocardial microvascular function even before the development of LVH and impairs myocardial perfusion responses to challenge. HT has been shown to impair the function of both the vascular endothelium and smooth muscle layers. For example, markers of endothelial dysfunction have been used to derive prognostic clinical information and monitor therapy. In addition, increases in arterial blood pressure induce proliferation of vascular smooth muscle cells and change their phenotype and sensitivity to and conductance of calcium. These observations denote the wide array of deleterious changes that HT provokes in the vascular wall.

The vascular endothelium also functions as a barrier, maintains homeostasis, and has anticoagulant and anti-inflammatory properties. Specifically, the barrier function of the vascular wall has been regarded as a parameter of vascular integrity and endothelial function. We have previously shown that cardiovascular risk factors like hypercholesterolemia can induce transient and dynamic increases in MVP. Similarly, it has long been recognized that HT is associated with alterations in MVP, as assessed by albumin extravasation. The current study extends previous observations and demonstrates noninvasively alterations in MVP in vivo, which might reflect impaired function of the endothelium. Their localization mainly in the subendocardial region might reflect its propensity for vascular dysfunction and myocardial ischemia, as previously suggested in clinical studies. Indeed, increases in MVP might not only reflect impaired vascular integrity but also play a role in vascular and myocardial changes.

In this study, we examined vascular function by using 2 common cardiac challenges, adenosine and dobutamine. Adenosine exerts its cardiac vasodilatory effect via specific A2 receptors, which are located on both the smooth muscle cells as well as the endothelium of small (<150 μm) myocardial microvessels. Therefore, adenosine-induced microvascular vasodilatation has both endothelium-dependent and -independent components, especially during intravenous administration, which is associated with flow-mediated vasodilation. Dobutamine, on the other hand, which exerts its effect by β-adrenergic receptor stimulation and by increasing the rate-pressure product, is not significantly endothelium dependent. Our observation that early HT impairs myocardial perfusion responses to both adenosine and dobutamine might implicate several potential mechanisms. The alterations in myocardial vascular function and MVP in HT in response to adenosine can be attributed to increased formation of reactive oxygen species, which are vasoconstrictors and can also blunt vasorelaxation by scavenging nitric oxide. Human and animal studies have demonstrated that HT is accompanied and paralleled by increases in oxidative stress. Thus, infusion of vitamin C was found to improve forearm vascular reactivity in hypertensive patients. In the current study, we used long-term dietary supplementation of a combination of vitamins E and C, which provides effective blockade of the endogenous oxidative stress cascade. Indeed, we observed that this regimen increased plasma vitamins levels and preserved endogenous scavenger enzyme activity, as we have previously shown in renal tissue, implying decreased abundance of superoxide anion in HT animals. Furthermore, myocardial perfusion and MVP responses to adenosine both improved.

Conversely, the attenuated response to dobutamine might result from HT-induced impairment in β-adrenoceptor-mediated vasodilatation or might reflect vascular remodeling. The greater improvement in the responses to adenosine than to dobutamine challenge in vitamin-treated HT might at least in part be related to their different mechanisms of action, because antioxidants are known to preferentially improve endothelium-dependent function. In contrast, the attenuated response to dobutamine might have been caused by the increase in blood pressure per se, which was not affected by antioxidant vitamins. Indeed, in the spontaneously hypertensive rat, long-term antioxidant treatment with melatonin or N-acetylcysteine, which in that study did decrease blood pressure, restored cardiac β-adrenoceptor function as well.

Our model of early HT is characterized by increased SVR and relatively mild elevations in arterial blood pressure, similar to those observed in clinical practice. Nevertheless, it...
was associated with a slightly reduced stroke volume and cardiac output. This is in agreement with the findings of Brin et al.,43 who showed that as soon as 3 months after induction of experimental HT, a decrease in cardiac output and stroke volume could be detected in dogs. The authors attributed that observation to differences in intracardiac compensation and regulation of heart function, but it might also have reflected an active cardiac remodeling process, possibly mediated by angiotensin II. Although systemic plasma renin activity was not increased in this model, local activation of the renin-angiotensin-aldosterone system cannot be excluded. The lack of cardiac hemodynamic improvement during antioxidant intervention might also imply a direct and sustained blood pressure effect, which was not affected by antioxidant intervention and chronically increased cardiac work. The present study is in agreement with prior observations44,45 that antioxidant intervention and chronically increased cardiac work. The present study is in agreement with prior observations44,45 that antioxidant intervention did not affect blood pressure. On the other hand, in several studies, antioxidant intervention did reduce blood pressures in HT.46–47 It is possible that antioxidant vitamins might improve some of the deleterious effects of oxidative stress (eg, endothelial function, lipid peroxidation, tissue injury),19 but might not succeed in reversing the deleterious effect of HT on other aspects (eg, vascular remodeling, vascular smooth muscle cell function, or nervous system activity). For example, the present study suggests that long-term antioxidant intervention does not preserve myocardial microvascular response to dobutamine. Differences in the effect of antioxidants on blood pressure might also respond to different doses, routes of administration, or timing and type of antioxidant intervention.68–50 The lack of change in cardiac output in early HT, as observed in other studies51 might be a result of different disease models used and different stages of the disease.

Blockade of oxidative stress might have significant implications in atherosclerosis.52–53 Both vitamin E and vitamin C are potent antioxidant vitamins, which prevent increases in oxidative stress15 and endothelial dysfunction.19,54 In the current study, we investigated an important functional effect of antioxidants on the myocardial microvasculature and show that daily dietary supplementation with a high-dose combination of both vitamins was effective in preserving myocardial perfusion and permeability responses to challenge in HT.

Perspectives
In this study, we demonstrate that long-term antioxidant intervention improves myocardial perfusion and MVP in early HT. These results suggest an important contribution of increased oxidative stress to myocardial microvascular dysfunction in early renovascular HT. However, their ability to impact more advanced stages of the atherosclerotic disease process remains to be established.

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References


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