Antioxidant Intervention Attenuates Myocardial Neovascularization in Hypercholesterolemia

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Background—Hypercholesterolemia (HC) and atherosclerosis can elicit oxidative stress, coronary endothelial dysfunction, and myocardial ischemia, which may induce growth-factor expression and lead to myocardial neovascularization. We tested the hypothesis that chronic antioxidant intervention in HC would attenuate neovascularization and preserve the expression of hypoxia-inducible factor (HIF)-1α and vascular endothelial growth factor (VEGF).

Methods and Results—Three groups of pigs (n=6 each) were studied after 12 weeks of normal or 2% HC diet or HC+antioxidant supplementation (100 IU/kg vitamin E and 1 g vitamin C daily). Myocardial samples were scanned ex vivo with a novel 3D micro-CT scanner, and the spatial density and tortuosity of myocardial microvessels were determined in situ. VEGF mRNA, protein levels of VEGF and VEGF receptor-1, HIF-1α, nitrotyrosine, and superoxide dismutase (SOD) were determined in myocardial tissue. The HC and HC+antioxidant groups had similar increases in serum cholesterol levels. HC animals showed an increase in subendocardial spatial density of microvessels compared with normal (160.5±11.8 versus 95.3±8.2 vessels/cm², *P*<0.05), which was normalized in HC+antioxidant (92.5±20.5 vessels/cm², *P*<0.05 versus HC), as was arteriolar tortuosity. In addition, HC induced upregulation of VEGF, HIF-1α, and nitrotyrosine expression and decreased SOD expression and activity, all of which were preserved by antioxidant intervention.

Conclusions—Changes in myocardial microvascular architecture invoked by HC are accompanied by increases in HIF-1α and VEGF expression and attenuated by antioxidant intervention. This underscores a role of increased oxidative stress in modulating myocardial microvascular architecture in early atherogenesis. (Circulation. 2004;109:2109-2115.)

Key Words: antioxidant ■ atherosclerosis ■ hypercholesterolemia ■ oxidative stress

Hypercholesterolemia (HC) is a major risk factor for atherosclerosis and ischemic heart disease, and even a relatively short exposure may impair myocardial perfusion.1 We have previously demonstrated that experimental HC was associated with coronary endothelial dysfunction1,2 that may lead to myocardial ischemia, which was partly mediated by increased oxidative stress3 and was preserved by chronic antioxidant supplementation.4 We have also shown that HC induced subendocardial neovascularization, most likely secondary to myocardial ischemia.5 However, the mechanism underlying this effect and the potential involvement of increased oxidative stress remained unclear.

A pivotal mechanism by which ischemia induces compensatory angiogenesis is through increased oxidative stress and consequently increased expression of the transcription factor hypoxia-inducible factor (HIF)-1α.6 HIF-1α may then upregulate the expression of vascular endothelial growth factor (VEGF),7 a potent angiogenic growth factor that stimulates endothelial cell proliferation and migration in vitro and angiogenesis in vivo.8,9 Furthermore, VEGF may be directly upregulated by oxidative stress intermediates such as superoxide radical10 or peroxynitrite,11 a pro-oxidant formed by the rapid reaction of superoxide and nitric oxide. Indeed, the increase in VEGF bioactivity in human epithelial cells exposed to superoxide can be blocked in vitro by antioxidants.10 However, it is as yet unknown whether chronic antioxidant supplementation in vivo can decrease VEGF expression and myocardial neovascularization.

Micro–computed tomography (micro-CT) is a novel and powerful imaging technique that permits assessment of the 3D pattern of the microvascular structure in situ12 and provides a unique and useful means for the study of the spatial distribution and connectivity of microvessels within an organ. We have demonstrated the feasibility of studying
myocardial microvascular architecture with micro-CT in pathophysiological states.\textsuperscript{5} Moreover, thanks to its ability to tomographically isolate single intramyocardial arterioles and their branches,\textsuperscript{12} it enables determination of microvascular tortuosity, a unique marker of vascular integrity and development of atherosclerosis.\textsuperscript{13}

Therefore, this study was designed to test the hypotheses that HC-induced changes in myocardial microvascular architecture (spatial density and tortuosity) would be attenuated during chronic antioxidant supplementation, in association with decreased expression of HIF-1\textalpha{} and VEGF.

Methods

All procedures using animals were reviewed and approved by the Institutional Animal Care and Use Committee. Three groups (n = 6 each) of age- and body weight–matched female domestic pigs (55 to 65 kg) were studied after 12 weeks of normal or HC diet (2% cholesterol and 15% lard by weight; TD93296, Harlan Teklad) or HC+dietary antioxidant supplementation (100 IU/kg of vitamin E and 1 g of vitamin C daily). We have previously shown that this combination is effective in decreasing oxidative stress and normalizing myocardial function.\textsuperscript{5,14} Plasma lipid profile (Roche) was determined after 12 weeks of diet in all 3 groups. Plasma prosta
glandin F\textsubscript{2}\alpha{}, isoprostane was determined by electrophoresisassay, as described previously.\textsuperscript{14} After euthanasia, the heart was removed for in vitro studies.

Micro-CT Procedure

An intravascular microfil silicone rubber (MV-122, Flow Tech, Inc), a contrast agent that essentially remains in the intravascular compartment, was used to perfuse through the cannulated left anterior descending coronary artery at a flow rate of 0.9 mL/min. A transmural portion of the left ventricular myocardium (\textapprox{}2\times{}1\times{}1 cm) was then sectioned, prepared, and scanned as described previously.\textsuperscript{5,15,16} Images were digitized for reconstruction of 3D volume images, which consisted of cubic voxels of 20 \mu{}m on a side and were displayed at 40 \mu{}m cubic voxels for subsequent analysis.\textsuperscript{5}

Images analysis was performed with the Analyze software package (Biomedical Imaging Resource). The myocardium was tomographically divided into 3 equal parts, and the data were analyzed in 7 slices obtained at equal intervals from each third. The outer two thirds of the myocardium were considered subepicardium, and the inner third was considered subendocardium.\textsuperscript{5} Using the “Object Counter” computer software, the spatial density of myocardial microvessels (diameters <500 \mu{}m) was calculated in each region.\textsuperscript{5,16}

Using a “connectivity” software that allows for tomographic isolation of a vessel, 1 to 3 intramyocardial arterioles and their branches were tomographically “dissected” in each pig, their branching pattern was visually assessed, and vessel elongation was determined by “Tree Analysis” software. For this purpose, the 3D path distance (total length) and linear distance (shortest distance between end points) of the main branches were calculated, and the elongation factor was calculated by dividing path distance by linear distance.

Spectrophotometry

CuZn-superoxide dismutase (SOD) and Mn-SOD were determined spectrophotometrically as described previously.\textsuperscript{5} All tissue activities were normalized for protein content by Lowry’s method.

RNA Isolation and cDNA Synthesis

Total RNA was isolated from myocardium by use of the TRIZOL (Invitrogen) method. RNA samples were purified with chloroform and isopropl alcohol, dissolved in diethyl pyrocarbonate (DEPC)–treated water, and quantified with a spectrophotometer (A\textsubscript{260}). cDNA was synthesized by use of the Invitrogen SuperScript first-strand synthesis kit as follows: 5 \mu{}g total RNA, 1 \mu{}L (10 mmol/L) dNTP mix, 2 \mu{}L (50 ng/\mu{}L) random-hexamers primers; the reaction volume was brought to 10 \mu{}L with DEPC water and incubated at 65°C for 5 minutes, followed by at least 1 minute of incubation on ice. Then, 2 \mu{}L 10X RT buffer, 4 \mu{}L (25 mmol/L) MgCl\textsubscript{2}, 2 \mu{}L (0.1 mol/L) diethiothreitol, 1 \mu{}L RNaseOUT, and 1 \mu{}L (50 U) of SuperScript II RT were added into the above reaction mixture and incubated at 25°C for 10 minutes, followed by 42°C for 50 minutes. The reaction was terminated at 70°C for 15 minutes, chilled on ice, and stored at −20°C.

Real-Time Quantitative PCR

To investigate the expression of VEGF mRNA, real-time polymerase chain reaction (PCR) (DNA engine OPTICON, MJ Research) was subsequently performed using the SYBR Green JumpStart Taq ReadyMix kit (Sigma). Briefly, 12.5 \mu{}L SYBR Green JumpStart Taq ReadyMix, 0.25 \mu{}L internal reference, 0.5 \mu{}L primer 5’ , 0.5 \mu{}L primer 3’, 1 \mu{}L cDNA, and 10.25 \mu{}L DEPC water reached 25 \mu{}L final reaction volume. The porcine gene–specific sequence of VEGF primers were used was upper, 5’-ACCAAGGCGGACGACATAG-3’ and lower, 5’-CTCGCTCTATCTTCTTTTGCTG-3’. The temperature profile included denaturation at 95°C for 3 minutes, followed by 45 cycles of denaturation at 95°C for 40 seconds, 60 seconds at 60°C annealing, and elongation with optics for 30 seconds, followed by 40 cycles of denaturation at 95°C for 40 seconds, 60 seconds at 60°C annealing, and elongation with optics for 30 seconds, followed by 40 cycles of denaturation at 95°C for 40 seconds, 60 seconds at 60°C annealing, and elongation with optics for 30 seconds. The relative amount of VEGF mRNA was normalized to an internal control GAPDH and relative to a calibrator (normal), calculated by 2\textsuperscript{−ΔΔCT}.\textsuperscript{17} The sequence of the GAPDH primer was upper, 5’-GGCCATGAACCATGAGAGT-3’ and lower, 5’-GTCTTCTGGGTCGCAAGAT-3’.

Western Blotting

Equal protein of myocardial homogenate was dissolved in SDS-polyacrylamide gels (10% or 15%) under reducing conditions (0.1 mol/L fresh dithiothreitol in sample buffer) and electrophoretically transferred onto polyvinylidene difluoride membrane (Bio-Rad).\textsuperscript{18} Membranes were blocked for 1 hour in Tris-buffered saline–Twee (TBST)/5% nonfat milk and incubated overnight at 4°C with antibodies against the 2 isoforms of SOD: Mn-SOD (1:200, Chemicon International) and CuZn-SOD (1:500, Santa Cruz Biotechnol) Antibodies against HIF-1\textalpha{} (1:200, Santa Cruz Biotechnology) and nitrotyrosine (1:500, Cayman Chemical) were also used. After washing with TBST, the membranes were incubated for 1 hour with horseradish peroxidase–linked anti-rabbit or anti-mouse antibody (1:5000, Amersham Pharmacia Biotech) in TBST/5% milk, and proteins were visualized by electrochemiluminescence. In additional experiments, to demonstrate specificity for nitrotyrosine, the membrane was incubated with 10 mmol/L dithionite (Sigma) for 1 hour at room temperature to remove tyrosine nitration.\textsuperscript{19} β-Actin (1:1000, Sigma) was used as the loading control.

ELISA Assay

VEGF and VEGF receptor-1 (Flt-1) protein levels were determined by ELISA (Human VEGF and Flt-1 kits, R&D Systems). Briefly, 100 \mu{}L of standards or serum was added to the wells of microplate precoated with a monoclonal antibody specific for human VEGF165 or Flt-1 and incubated for 2 hours at room temperature. The anti-VEGF antibody shows cross-reactivity with porcine, and validation studies have shown that this kit provides reliable measurement of VEGF concentration.\textsuperscript{20} After any unbound substances had been washed away, an enzyme-linked polyclonal antibody against VEGF or Flt-1 conjugated to horseradish peroxidase was added to the wells and incubated for 2 hours. Substrate solution was added to the wells and incubated for 30 minutes, and 50 \mu{}L of stop solution was then added for color development. The optical density of each well was determined with a microplate reader at 450 nm.

Statistical Analysis

Continuous data are expressed as mean±SEM. Multiple group comparisons used ANOVA, followed by unpaired t test, when applicable. Statistical significance was accepted at a value of P≤0.05. Protein expression was assessed relative to the loading control (actin) and expressed as ratio.
Results
After 12 weeks of HC diet, serum total and LDL cholesterol levels in HC and HC+antioxidant pigs were significantly and similarly higher than normal, whereas triglycerides remained unchanged (Table 1).

Intramyocardial Microvascular Architecture and Number
The vessels appeared more densely packed in HC than in normal hearts (Figure 1, top). Moreover, tomographically isolated microvascular trees in HC showed more ramifications and appeared more tortuous than in normal hearts (Figure 1, bottom). All of these alterations were preserved by antioxidant intervention. Quantitatively, the spatial density of small microvessels (<200 \( \mu \)m) was significantly higher in HC than normal (Table 2), an increase that was more pronounced in the subendocardium (ANOVA, \( P=0.007 \)), and was normalized in HC+antioxidants (Table 2).

Redox Status
Isoprostane levels were higher in HC, suggesting an increase in oxidative stress, but were significantly decreased in HC+antioxidant (Table 1). Compared with normal, HC pigs also showed decreased activity of both CuZn-SOD and Mn-SOD, implying blunted radical scavenging activity that remained attenuated in HC+antioxidant (Table 1). Conversely, protein expression of CuZn-SOD but not Mn-SOD was decreased in HC and also remained attenuated in HC+antioxidant (Figure 2). HC myocardium had increased immunoreactivity of nitrotyrosine, implying greater abundance of superoxide and interaction with nitric oxide, which was reduced in HC+antioxidant (Figure 3). Nitrotyrosine immunoactivity was abolished by pretreatment with dithion-

### Table 1. Systemic and Myocardial Tissue Characteristics of Normal, Hypercholesterolemic (HC), and HC+Antioxidant Pigs

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>HC</th>
<th>HC+Antioxidant</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cholesterol</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>2.1±0.1</td>
<td>9.6±2.1*</td>
<td>8.6±0.9*</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>0.3±0.0</td>
<td>0.4±0.1</td>
<td>0.2±0.0</td>
</tr>
<tr>
<td>HDL, mmol/L</td>
<td>0.9±0.1</td>
<td>2.7±0.6*</td>
<td>2.2±0.5*</td>
</tr>
<tr>
<td>LDL, mmol/L</td>
<td>0.9±0.2</td>
<td>6.8±1.7*</td>
<td>6.3±0.7*</td>
</tr>
<tr>
<td>Isoprostanes, pg/mL</td>
<td>98.7±8.7</td>
<td>152.7±9.8*</td>
<td>116.3±13.5</td>
</tr>
<tr>
<td>CuZn-SOD activity, mU/mg protein</td>
<td>8.0±0.7</td>
<td>5.8±0.1*</td>
<td>8.2±0.3</td>
</tr>
<tr>
<td>Mn-SOD activity, mU/mg protein</td>
<td>2.4±0.1</td>
<td>2.0±0.1*</td>
<td>2.4±0.1</td>
</tr>
<tr>
<td>Flt-1, pg/mg protein</td>
<td>1.8±1.0</td>
<td>2.0±0.7</td>
<td>1.9±0.9</td>
</tr>
</tbody>
</table>

*\( P<0.05 \) vs normal.

### Table 2. Spatial Density (vessels/cm\(^2\)) and Tortuosity (Elongation Factor, Dimensionless) of Myocardial Microvessels in Normal, Hypercholesterolemic (HC), and HC+Antioxidant Pigs

<table>
<thead>
<tr>
<th></th>
<th>Normal (n=6)</th>
<th>HC (n=6)</th>
<th>HC+Antioxidant (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Subendocardial, ( \mu )m</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤200</td>
<td>95.3±8.2</td>
<td>160.5±11.8*</td>
<td>92.5±20.5</td>
</tr>
<tr>
<td>201–300</td>
<td>8.4±3.0</td>
<td>9.1±1.7</td>
<td>4.1±0.9</td>
</tr>
<tr>
<td>301–500</td>
<td>3.0±0.9</td>
<td>5.3±1.6</td>
<td>2.9±0.7</td>
</tr>
<tr>
<td><strong>Subepicardial, ( \mu )m</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤200</td>
<td>97.2±14.4</td>
<td>157.7±18.5†</td>
<td>96.3±19.0</td>
</tr>
<tr>
<td>201–300</td>
<td>7.2±1.8</td>
<td>11.5±3.4</td>
<td>4.2±1.3</td>
</tr>
<tr>
<td>301–500</td>
<td>3.2±0.9</td>
<td>4.0±0.8</td>
<td>3.5±0.8</td>
</tr>
<tr>
<td><strong>Elongation factor</strong></td>
<td>1.06±0.02</td>
<td>1.39±0.10*</td>
<td>1.10±0.01</td>
</tr>
</tbody>
</table>

*\( P<0.05 \) vs normal.
†\( P=0.08 \) vs normal.

Figure 1. Representative 3D tomographic images (displayed at 40-\( \mu \)m voxel size) obtained from myocardium in normal, HC, and HC+antioxidant pigs. Top, Transmural myocardium. Bottom, Tomographically isolated transmural arterioles, showing in HC increased sprouting, which was prevented by antioxidant supplementation.
Myocardial Tissue

HC pigs showed increased expression of HIF-1α protein (Figure 4), which may reflect myocardial tissue hypoxia. VEGF mRNA and protein were also increased in HC (Figure 5), whereas the expression of its receptor Flt-1 remained unchanged (Table 1). All these parameters were preserved in HC animals that had been chronically supplemented with antioxidants (Figures 4 and 5).

Discussion

This study demonstrates that chronic blockade of the oxidative-stress cascade blunts HC-induced alterations in intramyocardial microvascular architecture and attenuates the expression of HIF-1α and VEGF.

Hypercholesterolemia is a major risk factor for coronary heart disease and may lead to coronary endothelial dysfunction and myocardial perfusion abnormalities. Furthermore, we have previously shown that HC is associated with microvascular neovascularization, possibly because of an increased subendocardial propensity for ischemia in microvascular disease. The present study demonstrates, for the first time, that diet-induced HC is also associated with increased vascular tortuosity. Increases in vascular tortuosity have been described previously in retinal, peripheral, and coronary arteries in association with advanced atherosclerosis, which might result from upregulation of elastolytic enzymes, breakdown of elastin fibers, and consequent vascular elongation and coiling, and their decrease by antioxidants in our study suggests a role for oxidative stress in this mechanism.

The mechanisms by which HC induces neovascularization may be multifactorial. Experimental HC is known to increase oxidative stress and inflammation, with consequent release of free radicals, cytokines, and growth factors. Hence, decreased SOD bioactivity and increased myocardial nitrotyrosine immunoreactivity in our HC pigs reflect attenuated scavenging activity and increased abundance of superoxide anion associated with a pro-oxidant shift. Both increased formation of superoxide radical and peroxynitrite further impair SOD activity and thereby lead to a vicious cycle of increased oxidative stress. Notably, decreased Mn-SOD enzymatic bioactivity is not necessarily accompanied by decreased Mn-SOD protein expression, which can in fact be paradoxically increased. Furthermore, the change in SOD bioactivity may be cell-type- and organ-specific, may show temporal variation during development of oxidative stress, and may depend on disease type, duration, and severity. In this study, although the activity of Mn-SOD was significantly decreased, its protein expression was not different from that in normal animals, supporting dissociation between the expression and functionality of this isoform. Indeed, disparate changes in the expression and function of these isoforms may be noted in disease states and reflect the diverse roles of this scavenger system.

This increase in oxidative stress has been shown to increase expression of HIF-1α, which in turn induces VEGF expression. Furthermore, oxygen-derived and other radicals are increasingly recognized as direct signaling me-
Diacritors both upstream and downstream to VEGF, and oxidized LDL per se can also directly increase VEGF expression. Therefore, increased oxidative stress may modulate neovascularization via several different pathways. Among these, hypoxia and increased VEGF expression are recognized as particularly potent angiogenic stimuli. Accordingly, this study shows that both VEGF mRNA and protein were increased in HC and normalized by antioxidant intervention. Although a decrease in VEGF protein expression could have been secondary to antioxidant-induced increased Flt-1 expression and VEGF binding, we observed that Flt-1 expression was similar in normal, HC, and HC+antioxidant pigs. These results support a direct effect of HC and antioxidants on the VEGF molecule rather than on its receptor. The present study also shows that chronic blockade of the oxidative stress not only preserved the expression of HIF-1α and VEGF but also normalized myocardial microvascular architecture in experimental HC. These results suggest an association between oxidative stress and neovascularization, which may be mediated in part via HIF-1α and VEGF. Antioxidants may attenuate this cascade at multiple points, and their beneficial effect may be a result of decreased LDL oxidation, inflammation, and/or redox-sensitive mechanisms and improved endothelial function and myocardial perfusion. Notably, we have previously shown that decreased myocardial perfusion in HC was inversely correlated with a concurrent increase in myocardial microvascular permeability, conceivably reflecting upregulation of VEGF, a potent vascular permeability factor. Remarkably, changes in microvascular permeability were not only normalized by use of antioxidant vitamin supplementation but significantly and inversely correlated with plasma concentrations of vitamins E and C and with tissue levels of vitamin E and Mn-SOD. These observations provide strong support to the relationship between myocardial ischemia (and thereby HIF-1α), VEGF, and increased oxidative stress.

The increase in vessel formation in HC might be a compensatory response aimed at sustaining myocardial perfusion. This response is probably only partly successful, because the newly formed vessels show abnormal function, and myocardial response to challenge remains impaired in HC. The ability of antioxidants to improve both myocardial microvascular architecture (as shown in the present study) and function (as we have shown previously) suggests that rather than interfering with a compensatory process, antioxidants blunt the upstream pathological stimulus (e.g., ischemia) that triggers adaptive structural changes in HC. Furthermore, this effect may be dose-dependent, similar to HMG-CoA reductase inhibitors, which have biphasic effects on angiogenesis. Indeed, the variable results of antioxidant vitamin intervention observed in clinical studies are most likely related to differences in study population, the duration, dose, and type of supplements, and outcome measures. Nevertheless, this capability of antioxidants to attenuate angiogenesis...
should be taken into consideration during design of therapeutic angiogenesis approaches.

Microvascular changes may occur during growth and maturation in the normal porcine heart. However, our previous studies showed that pigs similar to those used in the present studies have mature vasculature.\(^{41}\) In addition, all the pigs used in the present study were of similar age and body weight, arguing against interference of age- or weight-related changes with our results. Furthermore, our model of 12-week diet-induced HC does not induce left ventricular hypertrophy.\(^{42}\) A decrease in microvascular spatial density observed in antioxidant-treated animals may hypothetically be secondary to vasoconstriction or interstitial expansion, but these are unlikely to be induced by antioxidants, because our samples are allowed full relaxation before contrast injection.\(^{5}\)

Thus, we observed that increased HIF-1\(\alpha\) and VEGF expression induced by HC, with consequent changes in myocardial architecture, have been preserved by antioxidant intervention. Our study indicates that increased oxidative stress contributes to myocardial neovascularization in HC and implies a novel role for antioxidants in early atherogenesis.

**Acknowledgments**

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