Renal Vascular Function in Hypercholesterolemia Is Preserved by Chronic Antioxidant Supplementation

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Abstract. Hypercholesterolemia impairs systemic vascular reactivity in response to endothelium-dependent vasodilators, which may be mediated partly through increased formation of lipid peroxides. However, it is unclear whether these pathophysiological mechanisms play a role in renal vascular impairment in experimental hypercholesterolemia. Hence, pigs were studied after a 3-mo normal (n = 7) or high cholesterol (HC) (n = 7) diet, HC diet supplemented daily with antioxidant vitamins E (100 IU/kg) and C (1000 mg; HC+vitamins, n = 5), or normal diet supplemented with vitamins (N+vitamins, n = 5). Renal blood flow was measured with electron-beam computed tomography before and during infusion of acetylcholine (Ach). Endothelial function, endothelial and inducible nitric oxide synthase (NOS), and nitrotyrosine immunoreactivity were studied in renal arteries ex vivo. Despite similar cholesterol levels, LDL oxidizability (lag time, malondialdehyde, and relative electrophoretic mobility) was increased in pigs that were fed the HC diet but was significantly decreased in pigs that were fed the HC+vitamins diet. Renal blood flow response to Ach was blunted in pigs that were fed the HC diet but was preserved in pigs that were fed the HC+vitamins diet. Maximal relaxation to Ach was attenuated in pigs that were fed the HC diet compared with those that were fed the normal diet (51.5 ± 6.4% versus 97.0 ± 2.9%; P < 0.01) but was preserved in pigs that were fed the HC+vitamins diet (103.1 ± 3.0%; P = 0.39) and N+vitamins diet (87.7 ± 3.0%; P = 0.1), as were relaxation responses to calcium ionophore A23187. Vascular smooth-muscle relaxation to diethylamine was enhanced in endothelium-denuded HC vessel but was restored in pigs that were on the HC+vitamins regimen. In HC, immunoreactivity of endothelial NOS was decreased, that of inducible NOS was increased, and both were preserved in pigs that were fed the HC+vitamins and N+vitamins diets, whereas nitrotyrosine was not detected. The present study demonstrates that antioxidant intervention in experimental HC reduces LDL oxidizability and preserves renal vascular responses to endothelium-dependent vasodilators. Therefore, this beneficial effect potentially can protect the kidney from hypercholesterolemia-induced damage.

The role of the endothelium in regulation of vasomotor tone has been established during the past two decades. Impaired endothelium-dependent function of the systemic and coronary vasculature has been demonstrated in both animals and humans with overt atherosclerosis and with cardiovascular disease risk factors, such as hypercholesterolemia (HC) (1). One of the underlying mechanisms for impaired vascular reactivity is an increased release of oxygen radicals that react with nitric oxide (NO), thereby decreasing NO’s, bioavailability (2) and forming peroxynitrite. The impairment also likely is related to increased oxidizability of LDL, which impairs endothelium-dependent vascular dilatory responses (3,4) and is one of the pivotal steps involved in atherogenesis (5). Furthermore, oxidized LDL may affect NO bioavailability by modulating the expression of the enzyme endothelial NO synthase (eNOS) (6).

The vasculature of the kidney exhibits similar endothelial dysfunction in HC, both in vivo (7) and in vitro (8), and its consequences potentially may lead to renal damage and hypertension (9,10). Reactive oxygen species have been implicated in the pathogenesis of renal injury by direct cellular toxicity, partly through liberation of vasoconstrictor-bioactive lipids and inactivation of NO (11). In addition, oxidized LDL is injurious to renal tubular epithelial cells and may contribute to tubulointerstitial disease (12) and glomerulosclerosis (13).

Antioxidants such as vitamin E (tocopherol) and vitamin C (ascorbate) have been shown to attenuate the redox-sensitive mechanisms (14) and oxidative modification of LDL (15) in a mutually enhancing interaction (16). In the rat kidney, vitamin E attenuates the chronic renal injury associated with focal segmental glomerulosclerosis (17) and aging (18) and prevents renal interstitial fibrosis in hypercholesterolemia (19). However, the effects of chronic intervention with a combination of
antioxidant vitamins E and C on renal vascular function in hypercholesterolemia have not been defined clearly.

We showed previously that a 12-wk diet-induced HC in the pig was associated with impairment in renal vascular endothelial function (8). The present study was designed to explore the possible involvement of increased oxidative stress in this renal disturbance by reduction of redox-sensitive mechanisms with the use of chronic dietary antioxidant vitamin supplementation.

Materials and Methods

These studies were performed according to institutional guidelines for the care and use of laboratory animals. Domestic cross-bred pigs (60 to 70 kg) were fed for 12 wk a 2% HC diet (Harlan Teklad, Madison, WI) (7) (n = 4), an HC diet supplemented daily with oral vitamins E (100 IU/kg) and C (1000 mg; HC/H11001 n daily with the same doses of vitamins (N/H11001 a standard pig diet (normal, n = 2), noninsulin-dependent, and RBF was calculated as the sum of the products of (7,21,22). Cortical and medullary volumes then were calculated independently, and RBF was calculated as the sum of the products of the cortical and medullary perfusions and volumes (22).

EBCT Studies

For measurement of RBF, each animal was anesthetized with 0.5 g of intramuscular ketamine and xylazine, intubated, and mechanically ventilated with room air. Anesthesia was maintained with a mixture of ketamine (0.2 mg/kg per min) and xylazine (0.03 mg/kg per min) in normal saline, administered via an ear vein cannula at a rate of 0.05 ml/kg per min. Under sterile conditions and fluoroscopic guidance, intravascular catheters were placed in the suprarenal aorta and in the superior vena cava, as described previously (7,22). Mean arterial pressure was monitored throughout the experiment via the arterial catheter.

After tomographic localization of the midhilar section of both kidneys, 40 consecutive scans were obtained at variable time intervals (over 3 min) after a bolus injection (0.5 cc/kg over 1 s) of the nonionic, low-osmolar contrast medium iopamidol (Isovue 300 mg; Fort Dodge Laboratories, Fort Dodge, IA), which provides reliable measurements of RBF (7,21,22). The pigs then were killed with an intravenous overdose of pentobarbital sodium (Sleepaway, 30 mg/kg; Fort Dodge Laboratories, Fort Dodge, IA), and their kidneys were removed.

In Vitro Studies

The excised kidneys were immersed in cool, oxygenated physiologic salt solution (Kreb’s) of the following composition (in mM): 118.3 NaCl, 4.7 KCl, 1.2 MgSO4·7H2O, 1.22 KH2PO4, 2.5 CaCl2, 25.0 NaHCO3, 11.1 and glucose (control solution). Secondary or tertiary branches of the main renal artery (3 to 4 mm in diameter) were dissected from the kidneys under magnification and placed in control solution, with care taken to remove as much of the connective tissue as possible. Vessels of each animal were sectioned into rings 5 to 6 mm in length, some of which were preserved in 10% buffered formalin (Sigma Chemical Co., St. Louis, MO) or shock-frozen in liquid nitrogen and stored at −80°C. Additional rings were used for in vitro relaxation studies. In some rings, the endothelium was removed mechanically by insertion of the tip of a watchmaker’s forceps and scraping gently, a technique that has been described previously (23).

In Vitro Relaxation. Rings with and without endothelium (one from each animal) were suspended in 25-ml organ chambers filled with Kreb’s solution, maintained at 37°C, and aerated with 95% oxygen and 5% carbon dioxide (pH 7.4). Two stainless clips passed through the vessel lumen suspended each ring and were attached to a stationary post or to a strain gauge (Statham Gould UC 2; Viggo Spectamed Inc., Critical Care Division, Oxnard, CA) for measurement of isometric force. Rings were placed at the optimal point of their length-tension relationship by progressively stretching them until the contraction to potassium chloride (20 mM) was maximal. In all experiments, the presence of functioning endothelium was confirmed by response to Ach (10−6 M) after precontraction with potassium ions (20 mM) (8).

After optimal tension was determined and the presence or absence of endothelium was confirmed, the rings were allowed to equilibrate for 30 min before further experiments. All experiments were performed in the presence of indomethacin (10−5 M) to block endogenous production of prostaglandins. Endothelium-dependent relaxation after precontraction with endothelin-1 (ET-1; 10−7 M) was examined in one ring from each animal by use of increasing concentrations of Ach alone (10−9 to 10−5 M) or Ach in the presence of the NO synthesis inhibitor N0-monomethyl-L-arginine (L-NMMA; 10−4 M) and the endothelium-dependent vasorelaxant calcium ionophore A23187 (10−9 to 10−5 M).

To examine responses of the vascular smooth muscle, were exposed in five animals from each group endothelium-denuded vessels (one ring from each animal) to increasing doses of the NO donor diethylenetriamine (DEA; 10−7 to 10−5 M). At the end of the experiment, each ring was exposed to sodium nitroprusside (10−4 M) to establish a maximal relaxation response.

The effect of L-NMMA on attenuating responses to Ach (i.e., degree of relaxation attributable to endogenous NO) was calculated in each group by subtraction of the maximal Ach responses (at 10−5 M) of vascular rings preincubated with L-NMMA from those without preincubation (8).

The following drugs were used in conducting all experiments: Ach, calcium ionophore A23187, DEA, indomethacin, and sodium nitroprusside (Sigma); L-NMMA (Calbiochem, San Diego, CA); and ET-1 (Phoenix Pharmaceuticals, Inc., Belmont, CA). All powdered drugs were prepared with distilled water, and indomethacin was dissolved in Na2CO3. Stock solutions of each agent were prepared every day. Because of the buffering effect of the Kreb’s solution and aeration with 5% CO2, addition of L-NMMA did not alter organ bath pH. All drug concentrations are expressed as final concentration in organ chambers.

Cyclic Guanosine-3′,5′-Monophosphate Production. Cyclic guanosine-3′,5′-monophosphate (cGMP), the second messenger of NO, was measured to assess NO activity. One vascular ring from each of five animals in each group was placed in an organ chamber filled with Krebs solution. After 1 h of incubation, 146 µl of 3-isobutyl-
1-methyl-xanthine 10^{-4} M/L and 100 μL of indomethacin 10^{-5} M/L were added to the solution in the organ chamber for 30 min. Samples then were randomized to either standards (controls) or DEA (10^{-6} M/L) treatment for 1 min and then shock-frozen. The samples then were placed on dry ice and homogenized, and cGMP generation was measured on a scintillator counter, as described previously (8).

**Immunohistochemistry**

Immunoreactivity of eNOS and nitrotyrosine was examined on frozen 5-μm renal artery cross sections (one to two from each pig) mounted on positively charged slides. The slides were dried at 37°C for 1 h, fixed in acetone for 10 min at 4°C, and air dried for 60 min. Endogenous peroxidase activity was blocked by placing the slides in 1.5% hydrogen peroxide and 50% absolute methanol for 10 min and then rinsing. For eNOS staining, the slides were pretreated with 0.25% sodium dodecyl sulfate for 10 min and rinsed in tap water for 1 min, as described previously (24). For nitrotyrosine staining, the slides were not pretreated. The Vectastain Elite ABC Kit (Vector Laboratories, Inc., Burlingame, CA) for mouse IgG then was used, following the vendor’s instructions. Monoclonal antibodies to mouse eNOS (Transduction Laboratories, Lexington, KY), diluted 1:500 in phosphate-buffered saline (PBS), and to mouse nitrotyrosine residues (Cayman, Ann Arbor, MI), diluted 1:10 in PBS buffer, served as primary antibodies to the antigen. The tissue was stained by use of the Vector NovaRED substrate kit (Vector Laboratories, Inc.) for 10 min, followed by counterstaining with hematoxylin and mounting with aqueous mounting media.

Inducible NOS (iNOS) immunostaining was performed on 5-μm cross sections of the renal artery (two to three from each pig) cut from tissue embedded in paraffin blocks. The tissue was mounted on positively charged slides and dried at 37°C for 1 h. Before staining, the slides were deparaffinized in two changes of xylene, 5 min each, followed by two changes of 10 dips in absolute ethanol and two changes of 10 dips in 95% ethanol. Endogenous peroxidase activity was blocked by placing the slides in 1.5% hydrogen peroxide and 50% absolute methanol for 10 min and then rinsing. To block nonspecific binding sites, we subsequently incubated the tissue with 5% normal goat serum (Cayman, Ann Arbor, MI), diluted 1:100 in PBS buffer, served as primary antibodies to the antigen. The tissue was stained by use of the Vector NovaRED substrate kit (Vector Laboratories, Inc.) for 10 min, followed by counterstaining with hematoxylin and mounting with aqueous mounting media.

**Statistical Analyses**

Data are expressed as mean ± SEM. Vascular responses are expressed as percentage of change from the maximal preconstriction level of each renal arterial ring achieved with ET-1 (23). For RBF measurements, the results of both kidneys were compiled. Statistical evaluation of the data were performed with ANOVA or the t test for unpaired observations. Significance was accepted for $P < 0.05$.

**Results**

**Serum Cholesterol Levels and Systemic Hemodynamics**

At the end of the diet, the pigs that were on HC, HC+vitamins, normal, and N+vitamins diets had similar body weights (64.3 ± 3.2, 67.5 ± 4.9, 64.6 ± 1.2, and 63.3 ± 2.4 kg; $P = $ NS, ANOVA). Mean arterial pressure showed a trend for lower values in pigs that were on the HC diet, but this difference did not reach statistically significant levels (86 ± 6.9, 107.2 ± 7.0, 102.4 ± 7.0, and 111.2 ± 6.7 mmHg, respectively; $P = 0.06$). Serum lipids levels showed a statistically significant difference among the groups ($P = 0.00004$). Total and LDL cholesterol levels were significantly elevated in both groups that were fed an HC diet, compared with those on the normal diet (Table 1), but there was no difference in lipid fraction levels between vitamin-treated or -untreated pigs ($P ≥ 0.4$).

**LDL Oxidizability**

The susceptibility of LDL for oxidation was significantly increased in pigs that were on the HC diet, as evidenced by an increase in LDL-malondialdehyde and LDL–relative electrophoretic mobility and shortening of LDL lag time, compared with pigs that were on the normal diet (Table 1). Plasma levels of vitamins E and C in pigs that were on the HC diet tended to be lower than normal but were significantly increased in pigs that were on the HC+vitamins diet, compared with pigs that were on the normal and HC diets ($P < 0.001$; Table 1). In pigs that were on the HC+vitamins diet, LDL–relative electrophoretic mobility was significantly decreased and LDL lag time was significantly prolonged, compared with pigs that were on the normal diet ($P < 0.05$), and all markers for LDL oxidizability were significantly decreased, compared with pigs that were on the HC diet ($P < 0.01$ for all measurements). In pigs that were on the N+vitamins diet, plasma vitamins E and C were significantly increased and LDL oxidizability was further decreased, compared with those of the remaining three experimental groups.
Table 1. Serum lipid profile and measures of LDL susceptibility for oxidation in domestic pigs fed a 12-wk normal or HC diet, with and without daily oral supplementation with vitamins E (100 IU/kg) and C (1000 mg)\(^a\)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal (n = 7)</th>
<th>HC (n = 7)</th>
<th>HC + vitamins (n = 5)</th>
<th>Normal + vitamins (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>57.8 ± 3.0</td>
<td>449.2 ± 39.9(^b)</td>
<td>454.2 ± 67.2(^b)</td>
<td>83.0 ± 3.4</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dl)</td>
<td>19.0 ± 4.0</td>
<td>363.0 ± 35.2(^b)</td>
<td>360.3 ± 76.3(^b)</td>
<td>39.3 ± 3.6</td>
</tr>
<tr>
<td>Vitamin E ((\mu)mol/L)</td>
<td>59.5 ± 4.8</td>
<td>53.0 ± 3.2(^c)</td>
<td>167.5 ± 14.3(^b)</td>
<td>229.7 ± 17.5(^b)</td>
</tr>
<tr>
<td>Vitamin C ((\mu)mol/L)</td>
<td>95.8 ± 8.1</td>
<td>86.8 ± 2.7(^c)</td>
<td>133.3 ± 10.1(^b)</td>
<td>180.3 ± 8.4(^b)</td>
</tr>
<tr>
<td>LDL-MDA (nM/mg protein)</td>
<td>6.8 ± 0.4</td>
<td>8.7 ± 0.3(^b,c)</td>
<td>6.5 ± 0.3</td>
<td>3.2 ± 0.5(^b)</td>
</tr>
<tr>
<td>LDL-REM (mm from baseline)</td>
<td>11.1 ± 0.4</td>
<td>12.7 ± 0.5(^b,c)</td>
<td>9.9 ± 0.4(^b)</td>
<td>6.2 ± 0.6(^b)</td>
</tr>
<tr>
<td>LDL-lag time (min)</td>
<td>87.0 ± 5.3</td>
<td>74.0 ± 3.7(^b,c)</td>
<td>119.3 ± 9.0(^b)</td>
<td>131.3 ± 7.8(^b)</td>
</tr>
</tbody>
</table>

\(^a\) Measures of LDL oxidizability were obtained in four animals from each group. HC, hypercholesterolemic; MDA, malondialdehyde; REM, relative electrophoretic mobility (on agarose gel).

\(^b\) P < 0.05 compared with normal.

\(^c\) P < 0.01 HC versus vitamin-treated pigs.

**EBCT-RBF**

Under basal conditions, RBF was similar in pigs that were on the normal, HC, and HC + vitamins diets but tended to be higher in pigs that were on the N + vitamins diet (P = 0.07; Figure 1). Ach infusion did not induce a significant change in mean arterial pressure in any of the experimental groups (P = NS), as has been shown before (7). However, Ach induced a significant increase in RBF in pigs that were on the normal and HC + vitamins diets (P = 0.002 and P = 0.038, respectively) but not in pigs that were on the HC diet (P = 0.14; Figure 1). In pigs that were on the N + vitamins diet, RBF did not increase any further during Ach infusion but was similar to that observed in pigs that were on the normal and HC + vitamins diets under the same conditions and tended to be higher than that in pigs that were on the HC diet (P = 0.06).

**Vascular Endothelial Function**

The maximal contractile response to ET-1 achieved during precontraction was similar among the rings obtained from the different groups (P = 0.25), as were the maximal endothelium-independent relaxation response to sodium nitroprusside (10\(^{-4}\) M) achieved after each experiment (P > 0.25).

**HC Compared with Normal Diet.** Renal arterial rings removed from pigs that were on the HC diet showed a significantly attenuated maximal relaxation response to increasing doses of Ach (Figure 2A), compared with pigs that were on the normal diet (51.5 ± 6.4% versus 97.1 ± 2.9%; P < 0.0002). Relaxation in response to calcium ionophore A23187 also was attenuated in the pigs that were on the HC (Figure 2B) compared with the normal diet (72.8 ± 7.9% versus 107.9 ± 5.8%; P < 0.005). Preincubation of renal arterial segments with l-NMMA significantly blunted Ach-induced relaxation in the pigs that were on the normal (to 19.9 ± 8.5%; P < 0.0005 compared with normal diet without l-NMMA) and the HC (to 22.9 ± 8.1%; P = 0.02) diets, and their maximal relaxation to Ach after preincubation with l-NMMA was very similar (P = 0.8). However, the impact of l-NMMA on responses to Ach (i.e., degree of attenuation) was significantly greater in the pigs that were on the normal rather than the HC diet (77.1 ± 6.9% versus 29.1 ± 7.5%; P = 0.001; Figure 3), which suggests lower availability of endogenous NO in pigs that were on the HC diet.

**Effect of Antioxidant Vitamins.** Daily supplementation of vitamins E and C to the pigs that were on the HC diet normalized the maximal endothelium-dependent relaxation in response to increasing doses of Ach (103.1 ± 3.0%; P = 0.4 compared with pigs that were on the normal diet and P < 0.004 compared with rings from pigs that were on the HC diet; Figure 2A). Relaxation in response to calcium ionophore A23187 also was restored in pigs that were on the HC + vitamins diet (98.6 ± 5.2%; P = 0.3 compared with pigs that were on the normal diet) and was significantly

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**Figure 1.** Renal blood flow (RBF) measured with electron beam computed tomography in normal, hypercholesterolemic (HC) pigs (n = 7 animals each), HC pigs treated with antioxidant vitamins, and normal pigs treated with vitamins (n = 5 animals each). RBF was measured under basal conditions (■) and during infusion of acetylcholine (Ach; □). * P < 0.05 compared with baseline.
greater than that in pigs that were on the HC diet (Figure 2B; \(P < 0.005\)). After preincubation with l-NMMA, endothelium-dependent relaxation to Ach was significantly blunted in pigs that were on the HC+vitamins diet (to 8.9 \pm 1.4\%; \(P < 0.0001\) compared with vessels without preincubation) and was comparable to that in vessels of pigs that were on the normal diet (\(P = 0.25\); Figure 3). The impact of l-NMMA on attenuating responses to Ach (i.e., NO dependence) was significantly greater in pigs that were on the HC+vitamins than on the HC diet (94.6 \pm 5.3\%; \(P < 0.0001\) and was similar to that observed in vessels of pigs that were on the normal diet (\(P = 0.12\); Figure 3), which suggests restoration of endogenous NO activity.

In pigs that were on the N+vitamins diet, the maximal relaxation response to Ach was achieved at a lower dose of Ach and was similar to that of pigs that were on the normal and HC+vitamins diets (Figure 2A). At the highest Ach doses, the relaxation was attenuated (\(P = 0.02\) versus normal) but still was significantly greater than that in pigs that were on the HC diet (\(P = 0.03\)), and their relaxation response to calcium ionophore A23187 was similar to that of pigs that were on the normal diet (Figure 2B). The impact of l-NMMA on attenuating vascular responses to Ach in pigs that were on the N+vitamins diet was not significantly different from that observed in pigs that were on the normal or HC+vitamins diets (Figure 3), but it also was not significantly different from pigs that were on the HC diet.

**Vascular Smooth-Muscle Function**

Relaxation responses of endothelium-denuded arterial segments to the NO donor DEA were significantly enhanced in the pigs that were on the HC diet compared with rings from pigs that were on the normal diet (113.8 \pm 6.3\% versus 78.7 \pm 2.8\%; \(P = 0.0002\); Figure 4). Dietary supplementation with vitamins in the HC+vitamins group significantly diminished the enhanced response to DEA (88.7 \pm 2.6\%; \(P = 0.015\) compared with the HC group), although it still was greater than that in vessels of pigs that were on the normal diet (\(P = 0.04\); Figure 4). In pigs that were on the N+vitamins diet, the

**Figure 2.** Endothelium-dependent renal artery relaxation in normal (■, \(n = 7\)), HC (○, \(n = 7\)), vitamin-treated HC (●, \(n = 5\)), and vitamin-treated normal (×, \(n = 5\)) pigs in response to increasing doses of Ach (A) and calcium ionophore (B; A23187). *, \(P < 0.05\) compared with normal and vitamin-treated pigs.

**Figure 3.** Effect of preincubation with N\(^{G}\)-monomethyl-l-arginine (l-NMMA) on maximal relaxation of renal arterial segments in response to Ach (10\(^{-5}\) M). These were examined in rings removed from normal and HC pigs (\(n = 7\) each), HC pigs treated with antioxidant vitamins, and normal pigs treated with antioxidant vitamins (\(n = 5\) each). *, \(P < 0.05\) compared with normal arterial segments.
response to DEA was attenuated compared with that in the other groups (Figure 4).

Production of cGMP

Production of cGMP was similar among the groups under baseline conditions \((P = 0.35)\) and increased in all of the groups in response to DEA but to significantly different levels \((P = 0.0006)\). Vascular cGMP generation in response to DEA was augmented in pigs that were on the HC diet \((P < 0.01)\) compared with pigs on the normal diet; Figure 5), and concurrent vitamin treatment partly restored but did not normalize it \((P < 0.01); \text{Figure 5})\). In pigs that were on the N+ vitamins diet, cGMP generation was similar to that of pigs that were on the normal diet \((P = 0.4); \text{Figure 5})\).

Immunohistochemistry

The scoring of eNOS immunostaining in the renal arterial endothelium (Figure 6a) was lower in pigs that were on the HC diet \((1.9 \pm 0.3; P < 0.05)\) compared with those that were on the normal, HC+ vitamins, and N+ vitamins diets, all of which were similar to each other \((2.6 \pm 0.3, 2.8 \pm 0.6, \text{and} 2.8 \pm 0.4, \text{respectively}; P \geq 0.4)\). Immunostaining for iNOS was obvious in smooth-muscle as well as in endothelial cells of renal arteries obtained from pigs that were on the HC diet (Figure 6b) and was markedly increased compared with that of pigs that were on the normal diet \((3.0 \pm 0.2 \text{ versus} 1.3 \pm 0.2; P < 0.001)\). In pigs that were on the HC+ vitamins diet, iNOS immunoreactivity \((0.6 \pm 0.2)\) was greatly attenuated compared with that of pigs that were on the normal \((P = 0.01)\) and HC \((P < 0.0001)\) diets, whereas in pigs that were on the N+ vitamins diet, it was further attenuated \((0.2 \pm 0.1)\) compared with pigs that were on the normal and HC \((P < 0.0001)\) diets and tended to be lower than that in pigs that were on the HC+ vitamins diet as well \((P = 0.08)\). Nitrotyrosine immunostaining was undetectable in any of the experimental groups (Figure 6c).

Discussion

This study demonstrates that in experimental HC in the pig, dietary intervention with antioxidant vitamins E and C normalizes endothelium-dependent relaxation of the renal artery \textit{in vitro} and improves the abnormal renal vascular smooth-muscle response to exogenous NO. These effects are functionally consequential, given that they are associated with restoration of RBF response to challenge \textit{in vivo}. These studies suggest a role for activation of endogenous oxidation-sensitive mechanisms in renal endothelial dysfunction in HC.

Impairment in endothelium-dependent vasodilation of systemic and coronary vessels has been demonstrated consistently in HC, and we recently demonstrated (8) that the renal artery of HC pigs exhibited similar endothelial dysfunction. The functional significance of this abnormality is underscored by our recent observation that this deleterious effect extended to renal perfusion response \textit{in vivo} (7), as was also observed in the current study. This impairment potentially may eventuate in renal damage (9,10) and lead to chronic progressive renal injury. Alterations in vasomotor regulation may be responsible for enhanced propensity for vasoconstriction, and maladjusted RBF responses to physiologic challenges might result in repetitive renal insults (7).

In the current study, we observed that chronic treatment with a combination of vitamins E and C restored endothelium-
dependent relaxation of the renal artery of HC pigs. Experimental diet-induced HC resulted in blunted endothelium-dependent responses to both Ach and calcium ionophore A23187. Endothelium-denuded vessels from this group also displayed enhanced relaxation to the NO donor DEA, and cGMP production was increased. Determination of cGMP generation may not represent a direct measure of NO bioavailability (28), and this increase likely reflected a compensatory augmentation in arterial smooth-muscle sensitivity to NO (29,30), perhaps to offset partially the impaired endothelium-dependent vasodilation. After a 3-mo concurrent antioxidant vitamin treatment, endothelium-dependent relaxation in response to Ach and calcium ionophore was preserved and vascular response to DEA was almost restored, which suggests preservation of endogenous NO activity. However, although cGMP production was lower than in HC, it remained elevated compared with normal vessels. Hence, our study cannot exclude the possibility that the effect of antioxidants on vascular relaxation was in fact exerted partly through a direct effect on increased responsiveness of cGMP generation rather than on increased NO bioavailability alone. Nevertheless, the normalization of the vascular response of vitamin-treated HC pigs to L-NMMA and of eNOS protein expression in the same vessels support the role of the NO pathway in this abnormality.

Lower bioavailability of NO in HC may result from decreased expression and/or activity of eNOS (31), as well as from increased oxidative stress-mediated NO consumption. Endothelial superoxide anions produced in HC vessels interact with endothelium-derived NO and decrease its bioavailability (32) and are partly responsible for the impaired endothelial vasodilator function in HC (33). This interaction also can give rise to formation of peroxynitrite, a powerful oxidant that also can impair vascular function (34). Peroxynitrite subsequently can attack proteins and generate nitrotyrosine, which has been used as a marker of peroxynitrite reaction in vivo (35).

The absence of evidence for nitrotyrosine formation in our experimental groups underscores the importance of a balance between NO and reactive oxygen species in generation of peroxynitrite and in modulation of vascular pathophysiology. Similar to the vitamin E–deprived rat, in which abnormal vasorelaxation but not nitrotyrosine formation was observed (36), a role of peroxynitrite in our model with an early phase of HC may be more subtle than during evolution of more advanced atherosclerotic lesions (35). In conjunction with decreased eNOS expression in the renal artery and lower bioavailability of NO for interaction with the superoxide anion and despite the concomitant functional abnormality, peroxynitrite—and, consequently, nitrotyrosine—formation presumably may be detectable only after a longer exposure to the pro-oxidant milieu.

One of the mechanisms that are responsible for the decreased bioavailability of NO could be increased LDL oxidiz-

Figure 6. Representative immunostaining for endothelial NO synthase (eNOS; left panels), inducible NOS (iNOS; middle panels), and nitrotyrosine (right panels) in renal arteries of normal and HC pigs, with or without treatment with antioxidant vitamins. Immunoreactivity of eNOS was decreased in endothelial cells of HC pigs and restored in vitamin-treated pigs. Immunostaining for iNOS was markedly increased in smooth-muscle and endothelial cells of renal arteries obtained from HC pigs and was greatly attenuated in vitamin-treated pigs. Nitrotyrosine immunoreactivity was not detected in renal arteries of any of the experimental groups. Magnification, ×62.5.
ability, because oxidized (but not native) LDL is one of the regulators of eNOS (6) and thus affects NO synthesis (28). Incubation with oxidatively modified LDL also directly impairs endothelium-dependent vasodilation of the rabbit aorta (4). We showed recently that similar renal oxidation-sensitive mechanisms also were activated in renovascular hypertension and were accentuated when it coexisted with HC (37,38). Thus, diminishing oxidation of LDL may prove beneficial for vascular function. Indeed, the susceptibility of LDL for oxidation was increased in our HC group (38) and, despite similar plasma levels of LDL cholesterol, was significantly decreased in the vitamin-treated HC group in association with improvement in endothelial function.

In contrast to the decreased expression of the constitutive eNOS, immunoreactivity of iNOS was significantly increased in the renal artery of HC pigs. This isoform often is involved in proinflammatory processes (39) and can be found in inflammatory, smooth muscle, macrophages, and endothelial cells of human atherosclerotic lesions (40). In the kidney, it is expressed constitutively (41), but its maximal expression is induced by cytokines (42) in association with increased oxygen radical activity and renal injury (43,44) and locally inhibits eNOS (44). The current study shows that the renal artery shows increased iNOS immunoreactivity in early HC, which might represent an early stage of atherosclerosis preceding significant infiltration of inflammatory cells or development of vascular lesions. Mild basal iNOS immunoreactivity also was observed in the normal renal artery but was almost completely abolished in both vitamin-treated groups, likely because of the decrease in oxidative stress and/or LDL oxidizability, given that oxidized LDL is a strong stimulus for iNOS expression (45).

The large body of evidence that supports the important contribution of increased oxidation of LDL and endogenous oxidative stress to development of early atherosclerosis (46) has rendered antioxidant intervention an attractive therapeutic approach to preventing functional and structural vascular damage (14). As was reviewed recently (14,33), the efficacy of antioxidant treatment likely is related to the nature and timing of dietary supplementation. Vitamin E is the main lipid-soluble antioxidant in human plasma and lipoproteins, and it protects LDL against oxidation. Indeed, vitamin E supplementation to HC rabbits (47) and dogs (48) retarded the pathologic sequels of LDL oxidation and improved Ach-dependent vasorelaxation without affecting plasma cholesterol level. In humans, vitamin E decreases the susceptibility of LDL to oxidation (15,49).

Vitamin C, a potent aqueous-phase antioxidant in plasma, also seems to retard LDL oxidative susceptibility and contributes to regeneration of vitamin E (16), which is consumed during lipid peroxidation, thereby enhancing its effects.

The effects of antioxidant vitamins on vascular responsiveness in HC were less unequivocal. Dietary vitamin E improves systemic (50,51) and carotid (52) endothelium-dependent relaxation in HC rabbits. In the forearm of HC subjects, a 4-wk daily relatively high-dose therapy of vitamin E improved NO-related endothelial function (53), but the maximal response to Ach remained unchanged after 1 mo of therapy with vitamins E and C (49) or 8 wk of lower-dose vitamin E alone (54).

Because vitamin E may accumulate in tissues and cellular membranes, reaching levels 1000 to 100,000 higher than those usually administered in humans (14), the effects seen in the current study might have potential clinical interest. In support of this notion, we recently demonstrated that this interventional regimen improves myocardial perfusion response to increased cardiac demand in HC pigs (20).

Less is known about the effect of antioxidant vitamins on the renal vasculature in HC. In rats with chronic nephrosis accompanied by dietary HC, 32 wk of vitamin E supplementation in doses similar to those used in the current study attenuated renal injury (17), and 12 wk of vitamin E prevented renal interstitial fibrosis in uninephrectomized HC rats (19). Conversely, 4 wk of vitamin E supplementation to HC rabbits did not have a major effect on renal artery endothelial responses to Ach (52).

Hence, the favorable effects of vitamins on the kidney may also be dose, duration, and combination dependent. In support of this notion, Reckelhoff et al. (18) demonstrated that in kidneys of aging rats, a 9-mo high-dose vitamin E diet was more effective in the suppression of oxidative stress and the attenuation of the decline in renal function than a similar but lower-dose diet. Our study shows that high-dose antioxidant intervention has favorable effects on endothelium-dependent function of not only large renal vessels but also the intrarenal microvasculature, as indicated by improvement of RBF responses to Ach in vivo.

Although renal vascular functional responses both in vivo and in vitro in HC pigs that were supplemented with vitamins were very similar to those observed in normal pigs, responses in vitamin-treated normal pigs were, interestingly, somewhat attenuated. For instance, the sensitivity of endothelium-denuded vessels to DEA and NO dependence of vascular relaxation (responses to L-NMMA) were decreased, as were relaxation responses to high doses of Ach in vitro and RBF responses to Ach in vivo. This may be due to chronic, relatively high basal NO bioavailability, which also exceeded the dose of L-NMMA used. This postulation may suggest lower basal vascular tone and is supported by the observation that basal RBF of the N+ vitamins group tended to be high. Moreover, although it did not significantly increase in response to Ach, their RBF during Ach infusion was comparable to that observed in pigs in the normal or HC+vitamins groups. In addition, systemic levels of vitamins E and C in this group were higher than those in pigs that were on the HC+vitamins diet, possibly because of increased consumption of antioxidants in the latter group to counterbalance the pro-oxidant effects of the HC diet. Thus, with the high-dose intake and systemic levels of vitamins achieved in pigs that otherwise were fed a normal diet, these possibly manifested pro-oxidant properties, as has been suggested previously (16,47,55,56).

In summary, our results support the notion that antioxidant intervention improves renovascular endothelial function in HC. This may be mediated by decreased oxidation of LDL and by increasing bioavailability of NO, possibly through restored production and decreased consumption of NO by reactive oxygen species. Hence, these observations may suggest a ther-
apeutic role for antioxidant vitamins E and C for renal protection in HC.

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