Antioxidant vitamins induce angiogenesis in the normal pig kidney

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Divisions of 1Nephrology and Hypertension and 2Cardiovascular Diseases, Mayo Clinic College of Medicine, Rochester; 3Department of Biological Sciences, Minnesota State University, Mankato, Minnesota; 4The Research Center of Excellence in Cardiovascular Diseases and Departments of General Pathology and Medicine, University of Naples, Naples, Italy; and Evans Department of Medicine and Whitaker Cardiovascular Institute, Boston University, Boston, Massachusetts

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Daghini E, Zhu XY, Versari D, Bentley MD, Napoli C, Lerman A, Lerman LO. Antioxidant vitamins induce angiogenesis in the normal pig kidney. Am J Physiol Renal Physiol 293: F371–F381, 2007. First published April 11, 2007; doi:10.1152/ajprenal.00475.2006.—The effects of chronic supplementation with antioxidant vitamins on angiogenesis are controversial. The aim of the present study was to evaluate in kidneys of normal pigs the effect of chronic supplementation with vitamins E and C, at doses that are effective in reducing oxidative stress and attenuating angiogenesis under pathological conditions. Domestic pigs were randomized to receive a 12-wk normal diet without (n = 6) or with antioxidant vitamins supplementation (1 g/day vitamin C, 100 IU·kg−1·day−1 vitamin E; n = 6). Electron beam computed tomography (CT) was used to evaluate renal cortical vascular function in vivo, and micro-CT was to assess the spatial density and average diameter of cortical microvessels (diameter <500 μm) ex vivo. Oxidative stress and expressions of vascular endothelial growth factor (VEGF) and hypoxia-inducible factor (HIF)-1α were evaluated in renal tissue. The effects of increasing concentrations of the same vitamins on redox status and angiogenesis were also evaluated in human umbilical vascular endothelial cells (HUVEC). Compared with normal pigs, the density of cortical transmural microvessels was significantly greater in vitamin-supplemented pigs (149.0 ± 11.7 vs. 333.8 ± 48.1 vessel/cm², P < 0.05), whereas the cortical perfusion response to ACh was impaired. This was accompanied by a significant increase in tissue oxidative stress and levels of VEGF and HIF-1α. A low dose of antioxidant decreased, whereas a high dose increased, HUVEC oxidative stress and angiogenesis, which was partly mediated by hydrogen peroxide. Antioxidant vitamin supplementation can increase tissue oxidative redox and microvascular proliferation in the normal kidney, probably due to a biphasic effect that depends on basal redox balance.

antioxidative stress

ANGIOGENESIS, the process of developing of new vessels from preexisting vessels, is crucial for physiological organ growth and repair of wounds (4). However, excessive formation of new vessels has been associated with several pathological conditions, such as cancer (3, 10), inflammation (5), and atherosclerosis (20, 25), and has been shown to participate in disease progression (49). Interestingly, these conditions are all characterized by disequilibrium in redox status with increased production of reactive oxygen species (ROS). Although tissue hypoxia is the main drive for angiogenesis (18, 46), a growing body of evidence has demonstrated that oxidative stress can also be a potent trigger for the development of new vessels (24, 36, 38).

Overproduction of ROS can lead to activation of the hypoxia-inducible factor (HIF)-1 (52), which in turn regulates transcription of proangiogenic growth factors, including vascular endothelial growth factor (VEGF) (27, 39). Accordingly, previous human and animal studies have demonstrated that chronic supplementation with antioxidant vitamins E and C in cancer or in the presence of cardiovascular risk factors confers an antiangiogenic effect, resulting in decreased tumor mass growth (41) and improvement in vascular and organ function (5, 6, 55). On the other hand, our group and others have demonstrated that under normal conditions, when basal oxidative stress is not increased, antioxidant vitamins can have a paradoxical pro-oxidant effect (21, 48) and impair micro- and macrovascular function.

In the kidney, exposure to cardiovascular risk factors such as hypercholesterolemia increases tissue oxidative stress, impairs vascular endothelial and renal function (7) and induces renal neovascularization, which is most pronounced in the renal cortex (2, 5). Interestingly, chronic antioxidant vitamin supplementation in hypercholesterolemia blunted renal neovascularization, in association with a decrease in oxidative stress and renal damage (5). However, the effects of such a regimen of antioxidant vitamins on the microvascular architecture of the normal kidney, or the associated mechanisms, remain unknown.

Therefore, the aim of the present study was to evaluate in kidneys of normal pigs the effect of chronic supplementation with vitamins E and C, at doses that are effective for reducing oxidative stress and attenuating angiogenesis under pathological conditions. Moreover, to elucidate underlying mechanisms, redox status and angiogenesis were evaluated in human umbilical vascular endothelial cells (HUVEC) exposed to increasing concentrations of the same vitamins.

METHODS

In Vivo Study

This study was approved by the Institutional Animal Care and Use Committee. Twelve female domestic crossbred pigs (50–60 kg) were randomized to receive a normal diet without (n = 6) or with antioxidant vitamin supplementation (1 g/day vitamin C, 100 IU·kg−1·day−1 vitamin E; vitamin supplemented, n = 6) for 12 wk. Our group has previously shown that during increased oxidative stress associated with hypercholesterolemia or hypertension, this regimen is

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effective in restoring redox balance, decreasing neovascularization, and improving renal and myocardial vascular function (5, 44, 54, 55).

At the end of the study period, blood samples were collected, mean arterial pressure was measured using an intra-arterial catheter, and electron beam computed tomography (EBCT; Imatron C-150; Imatron, South San Francisco, CA) studies were performed to evaluate renal cortical vascular function. After euthanasia, performed with intravenous pentobarbital sodium (100 mg/kg, Sleepaway; Fort Dodge Laboratory, Fort Dodge, IA), the kidneys were immediately removed and prepared for micro-CT, as previously described (2, 9). In addition, pieces of the kidney and renal arterial branches were flash frozen in liquid nitrogen or preserved in formalin for subsequent tissue measurements.

**EBCT study.** EBCT studies were performed to assess basal and endothelium-dependent cortical vascular function. For this purpose, the pigs were anesthetized with intramuscular ketamine (20 mg/kg) and xylazine (2 mg/kg), and anesthesia was maintained with constant infusion of ketamine (0.2 mg·kg⁻¹·min⁻¹) and xylazine (0.03 mg·kg⁻¹·min⁻¹). Catheters were placed in the right atrium and aorta. One to two seconds before scanning, a bolus of the nonionic, low-osmolar contrast medium iopamidol (0.5 ml/kg over 2 s, Isovue-370; Bracco Diagnostics, Princeton, NJ) was injected into the right atrium, and 40 consecutive scans were obtained at the renal midhilar region (6-mm-thick slice) during a central venous infusion of iopamidol (0.5 ml/kg over 5 s), which highlights the renal cortex (6, 8, 9, 23, 53).

Images were reconstructed and analyzed using the Analyze software package (Biomedical Imaging Resource, Mayo Clinic, Rochester, MN). Regions of interest were manually traced in the aorta and the right and left renal cortex, and cortical perfusion (ml blood·min⁻¹·cm⁻³ tissue⁻¹) was then calculated, as previously described (6, 8, 9, 23, 53). Cortical volume was computed using a volume estimation program.

**Micro-CT procedure.** Kidneys were perfused ex vivo through a cannulated renal artery with 0.9% saline (containing 10 U/ml heparin) for 10–15 min, followed by an infusion of intravascular microfil silicone rubber (MV-122; Flow Tech, Carver, MA) infused at physiological pressure and a flow rate of 0.9 ml/min (2, 5, 9, 54). After complete polymerization, sections of microfil-filled renal cortex were prepared and scanned at 0.5° angular increments, using a micro-CT scanner. Three-dimensional volume images (cubic voxels of 20 μm on a side) were reconstructed and displayed at 40-μm cubic voxels for analysis (34, 55). With the use of Analyze software, the renal cortex was tomographically divided into three parts, starting at the juxtamедullary junction, which were separately analyzed as inner, middle, or outer cortex. In each region, cortical microvessels (diameter <500 μm) were counted, and their spatial density and average diameter were calculated (2, 5, 9, 53).

**Plasma analysis.** Vitamin E and C levels in plasma were determined using high-performance liquid chromatography (33). LDL oxidizability was assessed spectrophotometrically according to its lag time, malondialdehyde content (LDL-MDA), and relative electrophoretic mobility (LDL-REM) on agarose gel (0.8%), as previously described (29, 33), and thromboplastin-reactive substances (TBARS) were evaluated using spectrophotometry at 532 nm (19).

**Renal tissue analysis.** Renal tissue was analyzed using previously described methods. The levels of vitamins E and C in fresh frozen renal tissue were assessed using high-performance liquid chromatography, renal tissue oxidative stress was assessed according to the expression of nitrotyrosine by using immunostaining, and the levels of the radical scavenger enzymes CuZn superoxide dismutase (SOD), Mn SOD, and catalase were determined using spectrophotometry (8, 33, 44). The expression of NAD(P)H oxidase subunit p67phox, the oxidized LDL (ox-LDL) receptor LOX-1, endothelial nitric oxide synthase (eNOS) (all 1:200; Santa Cruz Biotechnology, Santa Cruz, CA), HIF-1α (1:100), matrix metalloproteinase (MMP)-2 (1:200; Santa Cruz Biotechnology), and MMP-9 (1:500; Chemicon International) were assessed using Western blotting with β-actin (1:500; Sigma, St. Louis, MO) as loading control. The production of superoxide anion was assessed using the oxidative fluorescent dye dihydroethidium (DHE) (6, 53), and an imaging program (MetaMorph) was used to calculate the ratio between red (oxidized) and blue (nonoxidized) fluorescence.

H₂O₂ production was evaluated using 2’,7’-dichlorodihydrofluorescein diacetate (DCHFDA; Molecular Probes, Eugene, OR) (51). Renal arteries were cut into 30-μm slices and exposed to 10 μM DCHFDA in PBS for 15 min at room temperature. Surface fluorescence was removed by washing the slice with PBS. The same imaging program was used for quantification of H₂O₂ after excitation at 480 nm and emission at 510 nm with an inverted fluorescent microscope (Nikon).

To assess angiogenic pathways, we measured renal protein expression of VEGF and basic fibroblast growth factor (bFGF) using ELISA and the expression of VEGF and HIF-1α using immunostaining (35). For quantification of immunostaining, cross sections of the kidney (1 per animal) were examined randomly using a computer-aided image analysis program (MetaMorph, Meta Imaging Series 4.6). In each representative slide, staining was semiautomatically quantified in 15–20 fields, expressed as a percentage of staining of total surface area, and the results from all fields were averaged (8). Vessel density (per cm²) was determined by averaging the number of vessels counted in six fields of each immunostained renal slide. Microvessels (<500 μm) were counted (×10) in α-smooth muscle actin (SMA)-stained slides (1:50; DAKO), and capillaries (~8 μm) were counted in von Willibrand factor (Biocare Medical, Concord, CA)-stained slides (×60).

**Cell cultures.**

**Tube formation.** To explore the effects of vitamins on microvessel formation in vitro, we cultured HUVEC (PromoCell, Heidelberg, Germany) with and without coinoculation with vitamin C (150 μM ascorbic acid; Sigma) and incremental doses of vitamin E (50, 100, 150, and 250 μM α-tocopherol; Sigma) (19) in BD BioCoat angiogenesis system (BD Biosciences, Bedford, MA). HUVEC (2 × 10⁴) were plated in each well and incubated at 37°C for 16 h with

<table>
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<th>Parameter</th>
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<th>Normal + Vitamins</th>
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<tr>
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<td>6</td>
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<tr>
<td>Body weight, kg</td>
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<td>Heart rate, beats/min</td>
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<td>LDL lag, min</td>
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<td>LDL REM, mm from baseline</td>
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<td>TBARS, nmol/ml</td>
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<td>Mn SOD, IU/mg protein</td>
<td>3.4 ± 0.1</td>
<td>4.7 ± 0.1*</td>
</tr>
<tr>
<td>Catalase, IU/mg protein</td>
<td>22.4 ± 1.5</td>
<td>34.5 ± 1.4*</td>
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LDL-REM, relative electrophoretic mobility of lipoproteins; MDA, malondialdehyde; TBARS, thiobarbituric acid-reactive substances; SOD, superoxide dismutase. *P < 0.01 vs. normal.
endothelial cell growth medium (Sigma). The formed tubes were then labeled with calcein AM (8 μg/ml; Molecular Probes) and counted using a fluorescence microscope, whereas tube length was measured using MetaMorph image analysis software.

In addition, the contribution of endogenous ROS to tube formation was evaluated using specific inhibitors of ROS formation. Superoxide anions are formed by single electron transfer to molecular oxygen, and their reaction with nitric oxide yields peroxynitrite. Instead, in aqueous solution, SOD catalyzes dismutation of superoxide to hydrogen peroxide (H₂O₂), which in turn is metabolized by enzymes like catalase to regenerate water and molecular oxygen. Therefore, parallel and similar in vitro experiments were performed in the presence of specific inhibitors of SOD (10 μM silver diethyldithiocarbamate; Sigma) and catalase (10 mM aminotriazole; Sigma) and in the presence of a higher concentration of ascorbic acid (300 μM; Sigma) as a scavenger of tocopheroxyl radicals.

Migration assay. HUVEC migratory function, which is essential for angiogenesis, was examined using a modified Boyden chamber technique (11). A 24-well Transwell apparatus (Costar) was used, with each well containing a 6.5-mm polycarbonate membrane with 8-μm pores. HUVEC (4 × 10⁴) were placed on the membrane, and the chamber was immersed in a 24-well plate, which was filled with endothelial cell basal medium-2 with 50 ng/ml of human VEGF₁₆₅. After incubation for 24 h, the membrane was washed briefly with PBS and the upper side of the membrane was wiped gently with a cotton ball. The membrane was then removed and stained with Giemsa solution. The magnitude of HUVEC migration was evaluated by counting the migrated cells in four random (×40) microscope fields.

Proliferation assay. HUVEC were seeded at 3 × 10⁵ cells/well in 96-well flat-bottom plates in endothelial cell growth medium-2 containing 2% FCS and allowed to adhere for 2 h. After 24 h of culture with or without concurrent exposure to different concentrations of vitamins, the proliferative activity was determined using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (CellTiter 96 nonradioactive cell proliferation assay; Promega, Madison, WI), which monitors the number of viable cells, according to vendor instructions (1). Briefly, MTS solution was added at 20 μl/well, and after 4 h of culture, the conversion of MTS to formazan was measured in a plate reader at 490 nm.

In situ detection of oxidative stress. Generation of ROS in HUVEC was measured with DHE and DCHFDA staining and fluorescence microscopy, following the manufacturer’s protocol. Briefly, RPMI 1640 without phenol red medium containing DHE (2 μM) or DCHFDA (10 μM) was applied onto each plate, incubated for 30 min in a light-protected humidified chamber at 37°C, washed once with...
PBS, and evaluated using inverted fluorescence microscopy. The effects of the inhibitors of catalase and SOD on superoxide and H$_2$O$_2$ production in HUVEC incubated with increasing vitamin E concentrations were also assessed.

**Growth factor expression.** Cells cultures were homogenized at 4°C in chilled protein extraction buffer. The homogenate was incubated in buffer for 1 h at 4°C, and the homogenized lysates were then centrifuged for 15 min at 14,000 rpm. The supernatant was removed, and protein concentration was determined by spectrophotometry using the Coomassie Plus protein assay (Pierce, Rockford, IL). The lysate was then diluted 1:4 in 1X PAGE sample buffer, sonicated, and heated at 95°C to denature the proteins. The lysate was then loaded onto a gel and subsequently run using standard Western blotting protocols with specific antibodies against VEGF (1:200, polyclonal rabbit; Santa Cruz Biotechnology) and HIF-1α (1:100, polyclonal rabbit; Santa Cruz Biotechnology), as well as β-actin (1:500, Sigma) as loading control. The membrane was exposed for 5 min to a chemiluminescent developing system (SuperSignal West Pico chemiluminescent substrate; Pierce), exposed to X-ray film (Kodak, Rochester, NY) and developed, and intensities of the protein bands were determined using densitometry. Protein expression was assessed relative to actin and expressed as a ratio.

**Statistical Analysis**

Results are means ± SE. Comparisons between experimental periods within groups were performed using paired Student’s t-test, and comparisons among groups were performed using analysis of variance (ANOVA). The dose-dependent effects of vitamin were compared using repeated-measures ANOVA. Statistical significance was accepted for $P \leq 0.05$.

**RESULTS**

**Effects of Antioxidant Vitamins In Vivo**

Systemic characteristics of the study groups are shown in Table 1. At the end of the diet period, the normal and vitamin-supplemented pigs had similar body weights, mean arterial pressure, heart rates, and lipid profiles, and no differences in serum creatinine were observed between the two groups ($P = 0.44$).

**Cortical volume and perfusion.** Cortical volume was similar in the normal and vitamin-supplemented pigs (89.6 ± 4.8 vs. 82.3 ± 5.3 ml, $P = 0.34$). Basal cortical perfusion was significantly higher in the vitamin-supplemented group compared with control pigs (5.6 ± 0.6 vs. 3.6 ± 0.2 ml·min$^{-1}$·ml$^{-1}$, $P < 0.05$). On the other hand, in normal animals, Ach induced a significant increase in the perfusion of the renal cortex (to 4.5 ± 0.5 ml·min$^{-1}$·ml$^{-1}$, $P < 0.05$ vs. baseline), whereas in vitamin-supplemented pigs, there was no
significant change in cortical perfusion in response to Ach [to 5.2 ± 0.3 ml·min⁻¹·m⁻¹, P = NS (not significant)].

Vascular architecture. The density of cortical microvessels (<500 μm) was significantly greater in vitamin-supplemented compared with control pigs (Fig. 1) in the inner (271.1 ± 35.3 vs. 129.3 ± 16.2 vessels/cm², P < 0.001), middle (351.9 ± 67.5 vs. 153.2 ± 13.3 vessels/cm², P < 0.001), and outer cortex (378.4 ± 51.3 vs. 164.5 ± 12.3 vessels/cm², P < 0.001). Moreover, the difference in transmural spatial density between the two groups was most evident in vessels <120 μm (Fig. 1).

Micro-CT data were confirmed by renal immunostaining. α-SMA staining (Fig. 2) showed a significant increase in the number of microvessels in vitamin-supplemented compared with normal pigs (749.5 ± 36 vs. 312.0 ± 28 vessels/cm², P < 0.001), and von Willebrand factor staining showed increased capillary density in this group (3.3 ± 0.2 × 10⁴ vs. 2.2 ± 0.2 × 10⁴ vessels/cm², P < 0.0001).

The increase in vascular density was accompanied by a significant increase in tissue levels of VEGF in vitamin-supplemented compared with normal animals (2.18 ± 0.6 vs. 1.11 ± 0.2 pg/mg protein, P < 0.05), whereas levels of bFGF were similar in the two groups (0.3 ± 0.04 vs. 0.3 ± 0.07 pg/mg protein, P = NS). The increased cortical expression of VEGF was also detected by immunostaining (26 ± 4 vs. 11 ± 1% area positively stained, P < 0.01, Fig. 2) and identified in both endothelial and tubular cells. Similarly, nuclear expression of HIF-1α by immunostaining was significantly greater in the cortex of vitamin-supplemented compared with normal pigs (4.0 ± 0.3 vs. 2.3 ± 0.3% area positively stained, P < 0.01, Fig. 2) and similarly distributed. Moreover, a significantly increased expression of MMP-2 and MMP-9 (Fig. 3) was consistent with angiogenesis in vitamin-supplemented animals.

Oxidative stress. At the end of observation, plasma vitamin C and vitamin E levels were significantly higher in vitamin-supplemented than control groups, as were renal tissue levels of vitamins E and C (Table 1), demonstrating the efficacy of supplementation. Plasma LDL oxidation and oxidizability were significantly reduced in the vitamin-supplemented group, with longer lag phase and lower LDL-REM and LDL-MDA than in controls; plasma TBARS remained unchanged (Table 1). However, the expression of the ox-LDL receptor LOX-1 was increased in renal vessels of vitamin-supplemented pigs (Fig. 3), indicating increased tissue uptake of ox-LDL.

In addition, DHE fluorescence showed a significant increase in local superoxide production, and DCHFDA staining showed an increased H₂O₂ level in renal vessels of vitamin-supplemented pigs (P = 0.05, Fig. 4). Similarly, nitrotyrosine immunoreactivity was significantly higher in the renal cortex of vitamin-supplemented compared with control pigs (8.4 ± 0.3 vs. 6.8 ± 0.6% area positively stained, P < 0.05, Fig. 4), and p67phox expression was increased (Fig. 3), suggesting increased tissue oxidative stress. Furthermore, decreased eNOS...
expression in renal arteries of vitamin-supplemented animals suggests a decreased ability to generate nitric oxide (Fig. 3). On the other hand, kidneys of vitamin-supplemented animals also showed increased activities of the radical scavenger enzymes SOD (both CuZn and Mn SOD) and catalase (Table 1). This may be a defensive mechanism, because tissue antioxidant enzymes initially respond to a challenge with pronounced upregulation, and the antioxidant defenses subsequently weaken (30, 45).

Effects of Antioxidant Vitamins In Vitro

Exposure to the lowest concentration of α-tocopherol (50 μM) was associated with a decrease in DHE and DCHFDA fluorescence (see Fig. 7), in parallel with decreased tube formation (Fig. 5) and decreased VEGF and HIF-1α expression (Fig. 6). Incubation with increasingly higher concentrations of α-tocopherol induced a progressive increase in oxidative stress (both superoxide anion and H2O2, Fig. 7), tube formation (Fig. 5), and VEGF and HIF-1α expression (Fig. 6). Increased HUVEC migration was observed in all concentrations of vitamin E starting at 100 μM (Fig. 5), whereas endothelial cell proliferation was significantly increased only at the 100 μM concentration alone (Fig. 5).

Coincubation with the catalase inhibitor aminotriazole, which reduces degradation of H2O2, significantly enhanced tube formation in response to vitamins in all doses (Fig. 6). On the contrary, coincubation with the SOD inhibitor diethyldithiocarbamate, which increases superoxide and decreases H2O2 abundance, significantly blunted tube formation in all doses of vitamin E (Fig. 6). Finally, doubling ascorbic acid concentration in the culture medium (to 300 μM) prevented the increased tube formation observed at the highest concentration of α-tocopherol (Fig. 6). Furthermore, HUVEC coincubated with high vitamin E concentration (>150 nM) and catalase inhibitor had higher DCHFDA staining but similar DHE staining, whereas cells incubated with the SOD inhibitor had increased DHE staining but similar DCHFDA staining, indicating the efficacy of the blockade of H2O2 and superoxide anion generation (Fig. 7).

DISCUSSION

The present study demonstrates that antioxidant supplementation in a therapeutic experimental dose that reduces renal microvascular proliferation associated with increased tissue oxidative stress contrarily elicits renal microvascular proliferation in the normal kidney, in which basal oxidative stress is
low. This is likely due to a biphasic, dose-dependent effect of vitamins E and C on angiogenesis, which may be partly mediated by H$_2$O$_2$.

The antioxidant effect of vitamins E and C is well established. However, studies both in vitro (22, 29) and in animal models (21, 48) have shown that antioxidant vitamins, particularly vitamin E, can have a paradoxical pro-oxidant effect when administered under conditions of normal or low basal oxidative stress. Indeed, the antiangiogenic effect of vitamin E has been observed in established tumors, which are characterized by hypoxia and oxidative stress, but not as a preventive measure (13). The dependence of the effects of antioxidant vitamins on preexisting redox status (43) suggests that their beneficial effect can be achieved mainly in conditions characterized by high basal oxidative stress but can be otherwise neutral or even detrimental in unselected subjects. Indeed, this dual effect could partially account for the controversial results of recent clinical trials on cardiovascular outcomes after chronic administration of vitamins in different populations (26).

Our group has previously shown that chronic administration of experimental doses of vitamins E and C improves renal (5, 44) and myocardial (32, 33) vascular function and decreases neovascularization (5, 54, 55) in hypercholesterolemia (5, 55) and hypertension (54). These disease conditions are characterized by decreased systemic basal levels of antioxidant vitamins (due to increased consumption) and increased oxidative stress, and therefore antioxidant administration effectively elevates vitamin concentrations to levels only slightly higher than normal. In contrast, in normal pigs, the same regimen was associated with excessive elevation of circulating vitamins E and C, increased oxidative stress, and impairment in myocardial microvascular and coronary artery endothelial function (48). The present study extends our previous findings and shows that in the normal pig kidney, this regimen of antioxidant vitamins increased oxidative stress, induced angiogenesis, and blunted renal endothelial function.

Moreover, we observed that chronic administration of vitamins E and C in pigs resulted in increased basal cortical perfusion, a phenomenon that was conceivably related to the higher number of cortical vessels, as demonstrated by the micro-CT, and associated with increased expression of VEGF and HIF-1$\alpha$ in the renal cortex. Since basal cortical perfusion was increased, activation of the HIF-1$\alpha$ pathway probably resulted from the increased tissue oxidative stress (37), rather than hypoxia, and triggered new vessel formation by upregulating renal expression of VEGF and MMP. Although both VEGF and bFGF have been implicated in angiogenesis, VEGF is the primary growth factor involved in ROS signaling (47) and in angiogenesis in the kidney (31), which may explain the unchanged expression of bFGF observed in this study in vitamin-supplemented animals. Indeed, the VEGF system does not necessarily require bFGF for exerting its angiogenic potential (16). The avid microvascular proliferation that resulted without a concomitant increase in renal size resembles that observed in the kidney of streptozotocin diabetic rats (50) and,
speculatively, may involve remodeling of the renal parenchyma. Indeed, we observed in vitamin-supplemented pigs increased expression of MMP-2 and MMP-9, which are implicated in degradation of extracellular matrix and likely allowed the newly formed vessels to grow in the kidney.

Despite the increase in vessel number, the blunted response to the endothelium-dependent ACh, reflecting endothelial dysfunction, suggests decreased availability of nitric oxide due to a shift in renal redox status, as shown by decreased eNOS expression in this study. The availability of nitric oxide likely also decreased by quenching by the superoxide anion. Indeed, despite improvement of the systemic markers of lipid oxidation, renal expression of nitrotyrosine was increased, suggesting protein nitration due to interaction of superoxide and nitric oxide, which might have also been derived from inducible NOS (15). Furthermore, increased fluorescence of DCHFDA in our study suggests increased levels of H2O2, which can upregulate VEGF in endothelial cells (12).

Interestingly, in line with other studies (21, 48), we observed dissociation between this pro-oxidant effect on the renal tissue and the antioxidant effect on plasmatic markers of lipid oxidation. It is plausible that whereas the lipid-soluble \( \alpha \)-tocopherol and the \( \alpha \)-tocopheroxyl radicals accumulate in the renal parenchyma, where they reach concentrations that allow the shift toward a pro-oxidant status (43), in the systemic circulation a potential pro-oxidant effect is limited by rapid liver elimination of the oxidized lipid products (40). Indeed, increased expression of LOX-1 in renal vessels may reflect increased propensity for renal uptake of ox-LDL.

To further investigate the mechanisms that may be responsible for the effect of antioxidant vitamins on the renal microcirculation, we studied the direct effect of increasing concentrations of \( \alpha \)-tocopherol (a preparation similar to that used in vivo) on HUVEC tube formation. Similar to the in vivo studies (5, 54, 55), low concentrations of the drug caused a decrease in cellular oxidative stress and VEGF and HIF-1\( \alpha \) expression.

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**Fig. 6.** Top: representative immunoblots and densitometry showing increased renal expression of VEGF (left) and HIF-1\( \alpha \) (right) in HUVEC incubated with vitamin C (150 \( \mu \)M) and \( \alpha \)-tocopherol at different concentrations (50, 100, 150, 250 \( \mu \)M). *\( P < 0.05 \) vs. vitamin E at 0 \( \mu \)M. Bottom: tube formation (number/field) dose-response to \( \alpha \)-tocopherol at similar concentrations, without and with coincubation with inhibitors of catalase or SOD (left) or doubling of ascorbic acid concentration (300 \( \mu \)M; right). *\( P < 0.05 \) vs. control. †\( P < 0.05 \) vs. 0 \( \mu \)M vitamin E.
and the parallel reduction in tube formation supports the role of ROS in stimulating the growth of new vessels. As the concentrations of α-tocopherol in the culture media increased, progressive accumulation of ROS, VEGF, and HIF-1α was likely responsible for the tube formation that increased proportionally. Interestingly, all concentrations of α-tocopherol induced endothelial cell migration, whereas endothelial cell proliferation was observed only at 100 μM, which might be the optimal concentration for this effect. Its selective effects on migration may therefore be comparable to the pro-angiogenic activity of β-carotene in HUVEC, which is exerted by activation of chemotaxis, cell adhesion, and matrix assembly, more than by HUVEC proliferation (14).

Our results suggest that α-tocopheroxyl radicals generated from α-tocopherol eventually assume an important role in the antioxidant-induced angiogenesis, although this was not independently confirmed under our experimental conditions.Doubling the concentration of ascorbic acid in the culture medium prevented the amplified angiogenic drive observed at high vitamin E concentration, probably by scavenging α-tocopheroxyl radicals (43). The importance of these radicals was also supported by the fact that in the presence of SOD and catalase inhibitors, increasing doses of vitamin E still succeeded to induce a progressive increase in tube formation. Rather, the inhibitor of catalase, which causes accumulation of hydrogen peroxide, evoked an upward shift of the dose-response curve to α-tocopherol, whereas the SOD inhibitor, which reduces H2O2, had an opposite effect. Notably, these effects were observed even in the absence of antioxidants, implicating H2O2 in HUVEC angiogenesis in vitro. Furthermore, our study demonstrated that high vitamin E concentration induced an increase of H2O2 level both in the kidney in vivo and in HUVEC in vitro. These data support the role of H2O2 as a mediator of angiogenesis both in vivo and in vitro, an effect that can

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conceivably be additive to that of α-tocopherol-derived radicals. Nevertheless, because of inherent differences between tube formation in vitro and angiogenesis in vivo, future studies are needed to determine whether H2O2 plays a similar role in antioxidant-induced angiogenesis in vivo.

Limitations and conclusions

In the present study we administrated to the pigs a single combination of vitamins E and C. The dose of vitamin E was considerably higher than the chronic supplementation usually given in human studies (400–800 IU) (42) but is common in experimental settings, whereas the dose of vitamin C (1 g/day) (17) has often been used in humans. Importantly, our group previously demonstrated that this dosage in pigs with hypercholesterolemia or hypertension, conditions characterized by increased oxidative stress, effectively improved renal oxidative stress, endothelial function, and microvascular architecture. The concentrations of vitamins E and C reached in the renal tissue are difficult to compare with the conventional doses of α-tocopherol and ascorbic acid used in vitro, but evidently this range covered both a decrease and an increase in angiogenesis, as observed in vivo.

In conclusion, the present study demonstrated that chronic administration of normal pigs with high-dose vitamins E and C can increase renal oxidative stress, activate the HIF-1-VEGF pathway, and lead to microvascular remodeling and cortical angiogenesis. This phenomenon was conceivably responsible for the increased cortical perfusion, whereas its blunted response to ACh can be attributed to endothelial dysfunction related to the increased oxidative stress. Moreover, the in vitro experiments demonstrated that the effect of vitamins on angiogenesis is related to their concentration, which in turn determines their pro/antioxidant effect, partly mediated by H2O2. Hence, this study underscores the need for adequate determination of basal oxidative stress before commencing treatment with high-dose antioxidant vitamins.

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