# Antioxidant Intervention Blunts Renal Injury in Experimental Renovascular Disease

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*Abstract.* Atherosclerotic renovascular disease (RVD) amplifies damage in a stenotic kidney by inducing pro-inflammatory mechanisms and disrupting tissue remodeling. Oxidative stress is increased in RVD, but its direct contribution to renal injury has not been fully established. The authors hypothesized that chronic antioxidant intervention in RVD would improve renal function and attenuate tissue injury. Single-kidney hemodynamics and function at baseline and during vasoactive challenge were quantified using electron-beam computed tomography in pigs after 12 wk of experimental RVD (simulated by concurrent hypercholesterolemia and renal artery stenosis, $n = 7$), RVD daily supplemented with antioxidant vitamins C (1 g), and E (100 IU/kg) (RVD+Vitamins, $n = 7$), or controls (normal, $n = 7$). Renal tissue was studied *ex vivo* using Western blot analysis and immunohistochemistry. Basal renal blood flow (RBF) and glomerular filtration rate (GFR) were similarly decreased in the stenotic kidney of both RVD groups. RBF and GFR response to acetylcholine was blunted in RVD, but significantly improved in RVD+Vitamins ($P < 0.05$ *versus* RVD). RVD+Vitamins also showed increased renal expression of endothelial nitric oxide synthase (eNOS) and decreased expression of NAD(P)H-oxidase, nitrotyrosine, inducible-NOS, and NF-κB, suggesting decreased superoxide abundance and inflammation. Furthermore, decreased expression of pro-fibrotic factors in RVD+Vitamins was accompanied by augmented expression of extracellular (matrix metalloproteinase–2) and intracellular (ubiquitin) protein degradation systems, resulting in significantly attenuated glomerulosclerosis and renal fibrosis. In conclusion, chronic antioxidant intervention in early experimental RVD improved renal functional responses, enhanced tissue remodeling, and decreased structural injury. This study supports critical pathogenic contribution of increased oxidative stress to renal injury and scarring in RVD and suggests a role for antioxidant strategies in preserving the atherosclerotic and ischemic kidney.

Renal artery stenosis (RAS), most commonly due to atherosclerosis (1), is an important clinical entity that can lead to hypertension and progressive renal failure (2). Clinical studies have consistently demonstrated that atherosclerotic renovascular disease (RVD) is particularly associated with greater deterioration of renal function as well as poorer responses to therapy compared with other causes of RAS (3). The mechanisms responsible for the increased propensity for renal damage have been proposed to include augmented generation of reactive oxygen species (ROS) and increased oxidative stress (4,5). ROS have been implicated in the pathogenesis of renal injury by liberation of vasoconstrictors, direct cellular damage, and inactivation of nitric oxide (NO) (6,7), which may subsequently lead to both functional and structural renal abnormalities. We have previously shown in a pig model that early RVD markedly increased oxidative stress, intrarenal inflammation, and fibrosis and amplified renal dysfunction (4,5). Evidence of potential benefits resulting from blockade of the oxidative stress pathway with antioxidant vitamins in renal disease is accumulating (8–10); however, their potentially beneficial effects on the stenotic kidney in atherosclerotic RVD have not been tested.

Electron-beam computed tomography (EBCT) is an ultrafast scanner, and we have previously shown that it provides accurate and noninvasive quantification of single kidney volume, regional perfusion, blood flow, GFR, and segmental tubular function (4,5,11–14). This clinically available technique provides an opportunity to repeatedly evaluate the regional renal hemodynamics and function of the intact RVD kidney *in vivo* distal to a stenosis and to define renal impairment and response to intervention. Thus, the goal of the present study was to test the hypothesis that blockade of the oxidative stress pathway by antioxidant vitamins in RVD would improve renal function and prevent intrarenal structural damage.

## Materials and Methods

All procedures were approved by the Institutional Animal Care and Use Committee. Twenty-one domestic pigs (55 to 65 kg) were studied

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after 12 wk of observation. In 14 pigs, a local irritant coil was placed in the main renal artery at baseline and induced gradual development of unilateral RAS, as described previously (4,5,12,14). BP measurement was obtained in all animals by use of a PhysioTel telemetry system (Data Sciences) implanted at baseline in the left femoral artery. Mean arterial pressure (MAP) was recorded at 5-min intervals and averaged for each 24-h period. Levels reported were those obtained for 2 d before each *in vivo* study (5,14). The degree of RAS was subsequently measured by quantitative renal angiography (4,12,14). To simulate early atherosclerosis, the animals were fed with either a high-cholesterol (HC) diet (2% cholesterol and 15% lard, TD-93296; Harlan-Teklad, Madison, WI) (4,5,13,15) (RVD, $n = 7$), or a HC diet orally supplemented with daily doses of vitamin C (1000 mg) and E (100 IU/kg) (10) (RVD+Vitamins, $n = 7$). We have previously shown that these doses and duration of treatment led to effective blockade of oxidative stress (10,14,16,17). The other seven pigs were fed with a normal diet and used as controls (normal, $n = 7$).

On study days, 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 (0.05 ml/kg per min). Under sterile conditions and fluoroscopic guidance, an 8F arterial catheter was advanced to the stenotic renal artery, proximal to the stenosis. Short bolus injections (4 to 6 ml) of low-osmolar nonionic contrast media (iopamidol, Isovue-370; Squibb Diagnostics, Princeton, NJ) were used to visualize the lumen of the renal artery using a fluoroscopy system (Siemens Siremobil Compact) and magnification that allows a field of view between 17 and 23 cm. The images were recorded and later analyzed off-line to determine the degree of stenosis. The degree of RAS was determined manually (using a ruler) by assessing the decrease in luminal diameter of the renal artery at the most stenotic point compared with a stenosis-free segment (12). After angiography, *in vivo* EBCT flow studies were then performed as previously detailed (4,13–15,18) for assessment of basal regional-renal perfusion, RBF, GFR, and tubular function and repeated during suprarenal infusion of acetylcholine (Ach, 5 $\mu$g/kg per min) and sodium-nitroprusside (SNP, 6 nM/kg per min) to test endothelium-dependent and -independent responses, respectively. Blood samples were collected from the inferior vena cava for measurement of vitamin levels (HPLC), plasma renin activity (PRA, radio-immunoassay), superoxide dismutase (SOD), activity and serum creatinine (spectrophotometry).

After completion of all studies, the pigs were euthanized with a lethal intravenous injection of Sleepaway (100 mg/kg intravenousl; Fort Dodge Laboratories, Inc, Fort Dodge, IA). Kidneys were removed using a retroperitoneal incision, immediately shock-frozen in liquid nitrogen, and stored at −80°C or preserved in formalin (5,13,14). *In vitro* studies were then performed to characterize oxidative stress and to assess pro-inflammatory and pro-fibrotic activity in the kidney. SOD activity was quantified in renal tissue and plasma using spectrophotometry. Protein expression of the NAD(P)H-oxidase subunits p67phox and p47phox, nitrotyrosine (as a footprint for peroxynitrite formation *in vivo*), endothelial nitric oxide synthase (eNOS), NF-$\kappa$B, matrix metalloproteinase–2 (MMP-2), and tissue inhibitor of metalloproteinase–1 (TIMP-1) were measured using Western blot analysis and immunohistochemistry. In addition, using either frozen or deparaffinized 5-$\mu$m-thick mid-hilar cross-sections, renal morphology (H&E), inflammation (ED-1 and CD-3), fibrosis (trichrome), as well as immunoreactivity for inducible NOS, TGF-$\beta$, and the intracellular protein degradation mediator ubiquitin were also investigated in representative slides from each kidney.

*Table 1.* Systemic characteristics, single-kidney hemodynamics (mean ± SEM), in normal, renovascular disease (RVD), and RVD treated with vitamins (RVD+Vitamins) pigs[a]

| | Normal ($n = 7$) | RVD ($n = 7$) | RVD+Vitamins ($n = 7$) |
|---|---|---|---|
| Total cholesterol (mmol/L) | 1.7 ± 0.1 | 8.4 ± 0.5[b] | 9.2 ± 1.2[b] |
| LDL cholesterol (mmol/L) | 0.6 ± 0.1 | 6.8 ± 0.6[b] | 6.8 ± 0.9[b] |
| Mean arterial pressure (mmHg) | 101.8 ± 4.4 | 123.8 ± 6.9[b] | 126.3 ± 4.3[b] |
| Degree of stenosis (%) | 0.0 ± 0.0 | 65.9 ± 9.5[b] | 65.8 ± 2.4[b] |
| Plasma renin activity (ng/mL per h) | 0.5 ± 0.1 | 0.4 ± 0.1 | 0.5 ± 0.1 |
| Creatinine ($\mu$mol/L) | 123.7 ± 6.2 | 176.8 ± 17.7[b] | 168.0 ± 8.8[b] |
| Vitamin E (mg/mmol LDL) | 1.2 ± 0.2 | 1.1 ± 0.2 | 2.5 ± 0.4[b] |
| Vitamin C (mg/L) | 0.4 ± 0.1 | 0.4 ± 0.1 | 0.65 ± 0.1[b] |
| Plasma SOD activity (U/ml) | 3.5 ± 0.3 | 1.7 ± 0.3[b] | 2.1 ± 0.4[b] |
| Renal volume (cc) | 142.5 ± 9.5 | 97.5 ± 10.4[b] | 78.8 ± 19.5[b] |
| Renal blood flow (ml/min) | 553.4 ± 48.7 | 306.6 ± 28.9[b] | 234.6 ± 69.1[b] |
| Glomerular filtration rate (ml/min) | 69.3 ± 4.4 | 50.0 ± 6.0[b] | 32.8 ± 12.1[b] |
| Perfusion (ml/min per cc) | | | |
| cortex | 4.2 ± 0.3 | 3.4 ± 0.3[b] | 3.3 ± 0.4[b] |
| medulla | 2.6 ± 0.3 | 2.3 ± 0.3 | 2.3 ± 0.4 |
| Intratubular fluid concentration (arbitrary units) | | | |
| proximal tubule | 3.8 ± 0.2 | 3.6 ± 0.4 | 3.7 ± 0.4 |
| Henle's loop | 8.2 ± 1.3 | 4.5 ± 0.7[b] | 6.5 ± 1.4 |
| distal tubule | 6.9 ± 0.9 | 5.0 ± 0.5[b] | 5.9 ± 0.9 |
| collecting duct | 11.3 ± 1.4 | 6.6 ± 0.8[b] | 6.5 ± 1.5[b] |

[a] LDL, low-density lipoprotein; SOD, superoxide dismutase.
[b] $P < 0.05$ *versus* normal.

### *Superoxide Dismutase Assay*

Total SOD, manganese (Mn), and copper-zinc (CuZn) SOD isoforms activity was measured in renal tissue using a Superoxide Dismutase Kit (R&D Systems, Inc, Minneapolis, MN), as described previously (14). For measurement of Mn SOD activity, 100 $\mu$l of potassium cyanide (KCN, 60mMol), which blocks CuZn SOD activity, was added to the mixture in parallel experiments.

In addition, SOD activity was measured in plasma using the Cayman Chemical Superoxide Dismutase Assay Kit (Cayman Chemicals, Inc, Ann Arbor, MI) and following the vendor's instructions. Briefly, blood anticoagulated with EDTA was centrifuged twice at 4°C, and the supernatant collected. The standards and samples were placed in a sample plate and assayed in duplicate. Reaction was initiated by adding 20 $\mu$l of diluted xanthine oxidase to all wells, and then the plate was incubated on a shaker at room temperature for 20 min. The absorbance of each standard and sample was read at 450 nm using a plate reader. SOD activity was calculated using an equation obtained from the linear regression of the standard curve substituting the linearized rate for each sample. One unit was defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical.

### *Protein Expression and Western Blotting*

Standard Western blot protocols were followed as described previously (5,13), using specific antibodies against nitrotyrosine residues (1:500; Cayman Inc., Ann-Arbor, MI), eNOS (1:250; BD Transduction Laboratories, Lexington, KY), the NAD(P)H-oxidase subunit p47phox (1:2000; Upstate Biotechnology, Lake Placid, NY); and p67phox, NF-$\kappa$B, MMP-2, and TIMP-1 (1:200 for all; Santa Cruz Biotechnology Inc., CA).

Immunohistochemistry for eNOS, iNOS, NF-$\kappa$B, CD-3, ED-1, nitrotyrosine, TGF-$\beta$, MMP-2, TIMP-1, and ubiquitin was performed on either frozen (eNOS) or deparaffinized renal tissue. Primary antibodies utilized were either polyclonal for iNOS (1:500; Affinity Bioreagents, Golden, CO), CD-3 (1:300; Cell Marque Corporation, AK), TGF-$\beta$ (1:10; Santa Cruz Biotechnology Inc., CA), TIMP-1 and PAI-1 (1:100 for both; Santa Cruz Biotechnology, CA), or monoclonal for ED-1 (1:50; RDI, NJ), eNOS (1:500; Transduction Laboratories), nitrotyrosine residues (1:20; Cayman, Ann-Arbor, MI), NF-$\kappa$B (1:50; Santa Cruz Biotechnology Inc., CA), MMP-2 (1:100; Chemicon International, Temecula, CA), and ubiquitin (1:200; Berkeley Antibody Co., Richmond, CA). The secondary antibody, IgG Envision Plus (Dako), was followed by staining with the Vector NovaRED substrate kit (Vector-Laboratories, Burlingame, CA), and slides were counter-stained with hematoxylin (4,5,13,14,19).

### *Data Analysis*

Manually traced regions of interest were selected in EBCT images in the aorta, renal cortex, medulla, and papilla, and their densities were sampled. Time-density curves were generated and fitted with extended gamma-variate curve-fits, and the area enclosed under each segment of the curve and its first moment were calculated using the curve-fitting parameters (11). These were used to calculate renal regional perfusion (ml/min per g of tissue), intratubular fluid concentration (ITC), which is an index of segmental tubular fluid reabsorption and tubular function, single-kidney GFR, and RBF, using previously-validated methods (4,11,14,15,18).

### *Histology*

Mid-hilar cross-sections of the ischemic kidney (1 per animal) were examined using a computer-aided image-analysis program (MetaMorph, Meta Imaging Series 4.6). In each representative slide, immunostaining was semi-automatically quantified in 15 to 20 fields by the computer program and expressed as percentage of staining of total surface area, and the results from all fields were averaged (4,5,13,14,19). Glomerular score (percentage of sclerotic glomeruli) was assessed by recording the number of sclerotic glomeruli out of 100 counted glomeruli (4,14).

### *Statistical Analyses*

Results are expressed as mean ± SEM. Comparisons within groups were performed using paired *t* test and among groups using ANOVA, with the Bonferroni correction for multiple comparisons followed by unpaired *t* test. Statistical significance was accepted for $P \leq 0.05$.

## Results

Serum lipids, MAP, and plasma creatinine were similarly and significantly elevated in both RVD and RVD+Vitamins

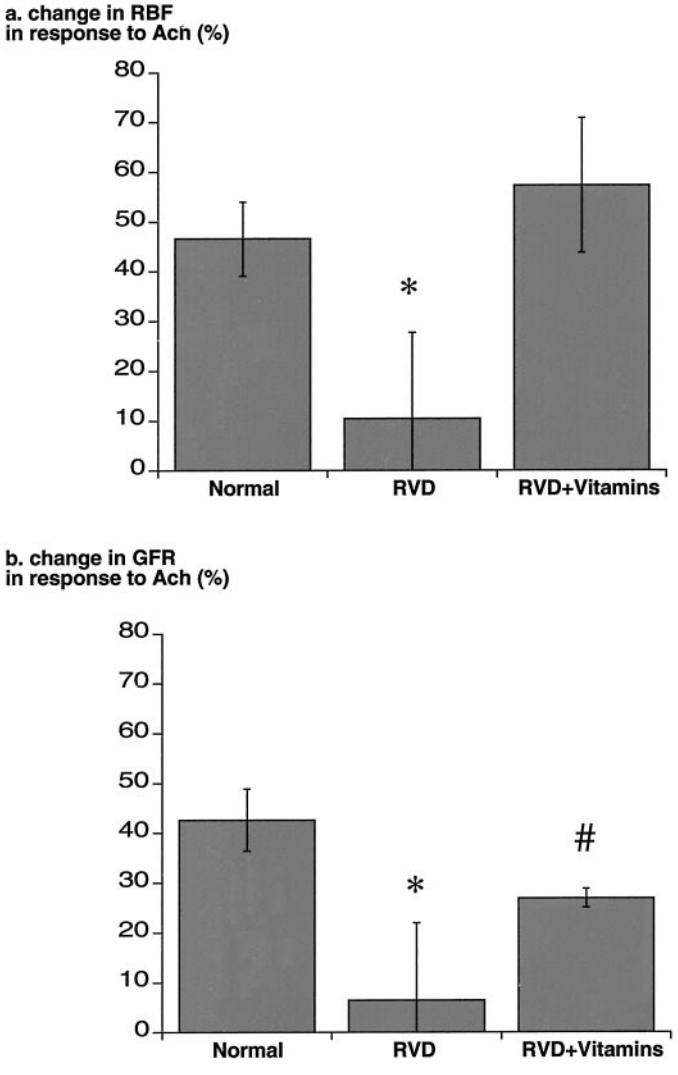


*Figure 1.* Renal blood flow (RBF) and GFR responses to acetylcholine (Ach, % change) in normal, renovascular disease (RVD), and RVD+Vitamins. Renal hemodynamics and function were significantly improved in RVD after antioxidant intervention. $^{*}P < 0.05$ *versus* normal, $^{\#}P = 0.09$ *versus* Normal.

compared with normal, and the angiographic degree of stenosis was not different between them (Table 1). PRA was not different among the groups (Table 1). Serum levels of vitamin C and E were significantly increased in the treated animals, while systemic plasma SOD activity was attenuated in both RVD groups (Table 1).

### *Basal Renal Hemodynamics and Function*

Basal renal volume, RBF, GFR, and cortical perfusion were significantly decreased in RVD and RVD+Vitamins compared with normal (Table 1), while medullary perfusion was similar among the groups. Compared with normal animals, ITC was significantly lower along the nephron of RVD from the loop of Henle and distally, suggesting impaired tubular fluid reabsorption, as previously shown (4). This impairment was mostly reversed in RVD+Vitamins, although it remained unaltered in the collecting duct (Table 1).

### *Response to Acetylcholine and Sodium-Nitroprusside*

Infusion of Ach and SNP was not associated with a persistent change in BP, as we have previously shown (4). In normal animals, Ach significantly increased RBF and GFR (to 737.1 ± 56.1 and 97.4 ± 8.3 ml/min, respectively; $P \leq 0.003$ for both), cortical and medullary perfusion (to 5.7 ± 0.5 and 4.2 ± 0.4, ml/min per g, respectively; $P \leq 0.02$ each). This response was blunted in RVD, in which Ach did not further increase RBF, GFR, or any regional perfusion compared with baseline. However, in RVD+Vitamins, Ach significantly increased single-kidney RBF (to 318.4 ± 73.3 ml/min; $P = 0.01$), GFR (to 48.3 ± 16.9 ml/min; $P = 0.03$), and cortical perfusion (to 4.7 ± 0.3 ml/min per g; $P = 0.02$). Furthermore, the degree of RBF and GFR responses to Ach was greater in RVD+Vitamins compared with RVD (57.4 ± 13.7 *versus* 22.7 ± 18 and 26.8 ± 1.8 *versus* 6.4 ± 15.6% change; $P < 0.05$; Figure 1). Medullary perfusion remained unchanged.

In response to SNP, normal animals showed a significant increase in RBF (to 667.9 ± 57.8 ml/min; $P = 0.04$), cortical, and medullary perfusion (to 4.9 ± 0.4, and 3.6 ± 0.39 ml/min per g of tissue, respectively; $P < 0.04$ for both), whereas these remained unchanged in RVD and RVD+Vitamins.

### *Redox Status*

The activity of both CuZn and Mn SOD isoforms was significantly decreased in RVD compared with normal and remained attenuated in RVD+Vitamins (Table 2), suggesting that antioxidant intervention did not improve SOD-mediated superoxide anion scavenging. However, the increased protein expression of both the NAD(P)H-oxidase subunits p67phox and p47phox observed in RVD was normalized in RVD+Vitamins (Figure 2, A and B), suggesting decreased potential for generation of superoxide. Furthermore, tubular and glomerular protein expression of nitrotyrosine, which was significantly elevated in RVD kidneys compared with normal, was substantially reduced in RVD+Vitamins (Table 2, Figure 2C), implying decreased production of peroxynitrite.

### *Renal Inflammation and Fibrosis*

Microvascular endothelial expression of eNOS was not different than normal in RVD animals but showed a strong tendency to increase in RVD+Vitamins ($P = 0.08$ *versus* RVD; Table 2 and Figure 3A), suggesting increased bioavailability of nitric oxide (NO). In addition, tubular (mainly proximal) and glomerular expression of the pro-inflammatory fac-

*Table 2.* Renal tissue redox status (mU/mg, mean ± SEM), protein expression, and morphological evaluation (% of renal area; mean ± SEM), in normal, renovascular disease (RVD), and RVD treated with vitamins (RVD+Vitamins) pigs

| | Normal ($n = 7$) | RVD ($n = 7$) | RVD+Vitamins ($n = 7$) |
|---|---|---|---|
| Tissue SOD activity (mU/mg) | | | |
| Total | 76.7 ± 5.2 | 42.9 ± 8.7[a] | 47.3 ± 8.4[a] |
| CuZn | 67.8 ± 3.9 | 42.2 ± 8.4[a] | 44.8 ± 7.1[a] |
| Mn | 8.9 ± 1.9 | 0.7 ± 0.4[a] | 2.2 ± 1.8[a] |
| Nitrotyrosine | 7.5 ± 0.3 | 10.4 ± 0.5[a] | 3.8 ± 0.6[ab] |
| eNOS | 4.4 ± 0.3 | 3.9 ± 0.4 | 5.6 ± 0.6[c] |
| iNOS | 3.4 ± 0.4 | 11.8 ± 0.4[a] | 5.0 ± 0.1[ab] |
| NF-$\kappa$B | 0.4 ± 0.06 | 6.7 ± 0.9[a] | 0.7 ± 0.04[ab] |
| TGF-$\beta$ | 1.5 ± 0.09 | 7.1 ± 0.5[a] | 3.1 ± 0.3[ab] |
| TIMP-1 | 2.0 ± 0.1 | 6.2 ± 0.4[a] | 3.7 ± 0.5[ab] |
| MMP-2 | 3.4 ± 0.1 | 3.3 ± 0.2 | 3.8 ± 0.1[ab] |
| Ubiquitin | 3.1 ± 0.2 | 4.3 ± 0.4[a] | 6.3 ± 0.4[ab] |
| Trichrome | 3.3 ± 0.2 | 8.6 ± 0.4[a] | 6.8 ± 0.6[ab] |
| Glomerular score | 0.0 ± 0.0 | 4.0 ± 1.1[a] | 1.9 ± 0.3[ab] |

[a] $P < 0.05$ *versus* normal.
[b] $P < 0.05$ *versus* RVD.
[c] $P = 0.08$ *versus* RVD.

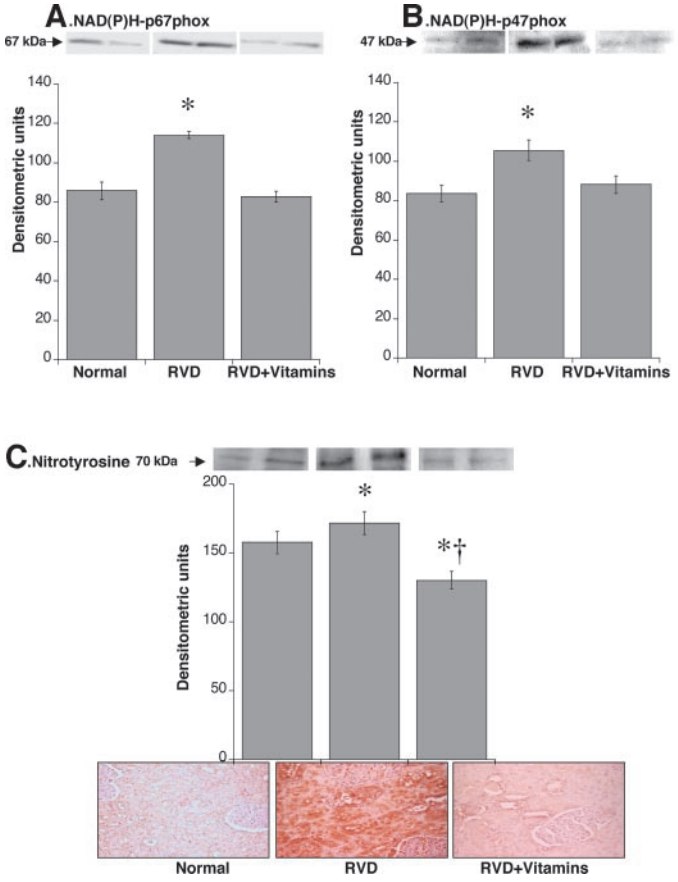


*Figure 2.* Renal expression (cortex and medulla) of NAD(P)H-p67 phox (A), p47phox (B), and nitrotyrosine (C) in normal, renovascular disease (RVD), and RVD+Vitamins kidneys. Vitamin supplementation in RVD normalized the expression of NAD(P)H-p67phox, p47phox, and nitrotyrosine, suggesting decreased superoxide generation and peroxynitrite formation. *$P < 0.05$ *versus* normal, †$P < 0.05$ *versus* RVD. Magnification, ×40.

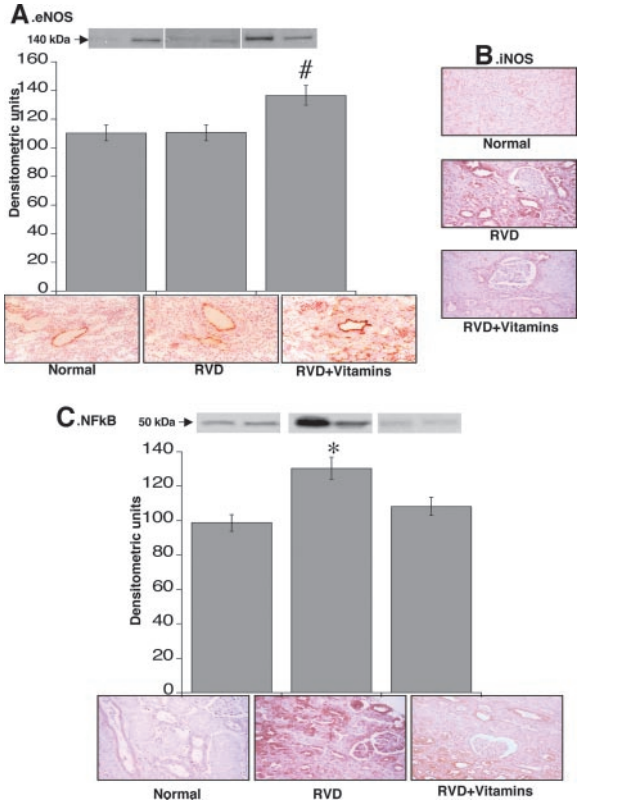


*Figure 3.* Representative renal immunoblots and immunohistochemistry demonstrating protein expression of eNOS (A) and pro-inflammatory iNOS (B) and NF-κB (C) in normal, renovascular disease (RVD), and RVD+Vitamins kidneys. Chronic antioxidant supplementation in RVD increased eNOS expression and decreased iNOS and NF-κB, suggesting augmented generation of NO and a decrease in inflammation. *$P < 0.05$ *versus* normal, #$P = 0.08$ *versus* RVD. Magnification, ×40.

tors iNOS and NF-κB, which was markedly elevated in RVD animals, were substantially decreased in the vitamin-treated group (Table 2; Figure 3, B and C), indicating attenuation in renal inflammation. Furthermore, the expression of the pro-fibrotic TIMP-1 and TGF-$\beta$ was increased in the tubular and glomerular compartments of RVD compared with normal, accompanied by a marked increase in perivascular, glomerular, and tubulointerstitial fibrosis. In contrast, kidneys from animals treated with vitamins showed a significant decrease in TIMP-1 and TGF-$\beta$ expression (Table 2; Figure 4, A and B), suggesting decreased inclination for matrix deposition. Furthermore, the expression of MMP-2 and ubiquitin was elevated in RVD+Vitamins ($P < 0.02$ *versus* normal and RVD for both, respectively; Table 2 and Figure 4, B and C), implying an increase in extracellular and intracellular protein degradation and removal. Accordingly, RVD animals showed a marked increase in $CD\text{-}3^+$ and $ED\text{-}1^+$ cells (Figure 5, A and B), which were normalized after antioxidant intervention. Consequently, although it remained greater than normal ($P < 0.001$ *versus* normal; Table 2), the trichrome histopathological analysis of stained sections illustrated a significantly reduced glomerular score (an index of glomerulosclerosis) in RVD+Vitamins, accompanied by diminished tubular and interstitial staining ($P = 0.02$ *versus* RVD; Table 2), suggesting an overall decrease in renal fibrosis (Table 2; Figure 5C).

## Discussion

This study demonstrates that chronic blockade of the oxidative stress pathway in an experimental model of early RVD improves RBF, GFR, and regional perfusion responses to challenge and blunts inflammation and fibrosis in the stenotic kidney. Thus, antioxidant vitamin intervention might play a role in preserving the function and structure of the stenotic kidney and delaying progression of renal injury.

Atherosclerotic RVD is associated with high cardiovascular morbidity and mortality and has been increasingly recognized as a major cause of end-stage renal disease (20,21). The augmented renal parenchymal damage observed in atherosclerotic RVD is considered a major determinant of renal dysfunction and outcome (22). Inhibition of NO-mediated vasodilatation, activation of angiotensin II, increased generation of ROS, and modulation of cell growth and proliferation may mediate renal

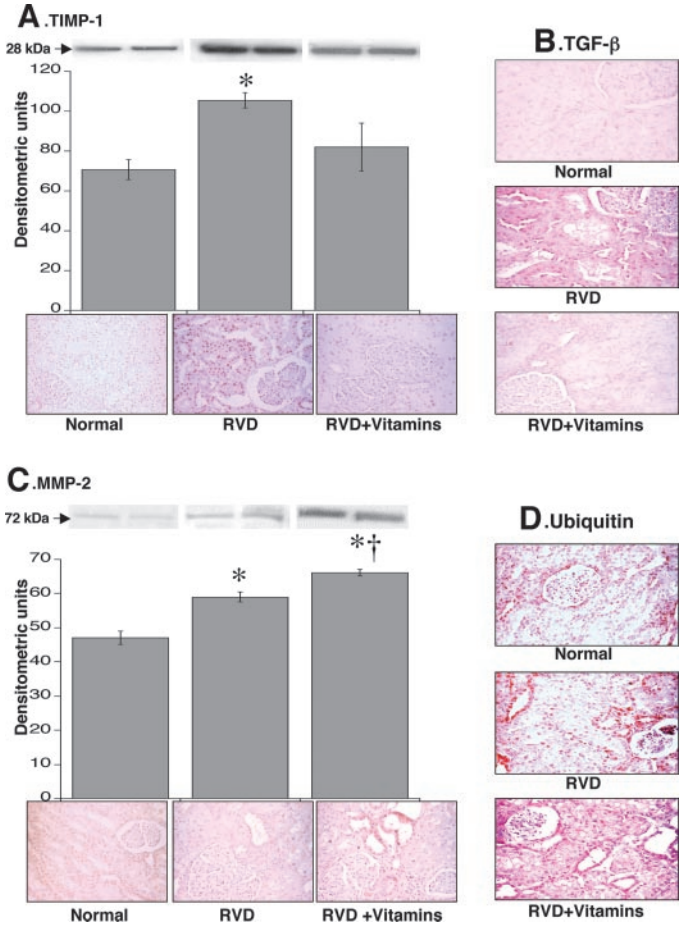


*Figure 4.* Representative renal immunoblots and immunohistochemistry demonstrating protein expression of tissue inhibitor of metalloproteinases–1 (TIMP-1) (A), TGF-$\beta$ (B), matrix-metalloproteinase–2 (MMP-2) (C), and ubiquitin (D), in normal, renovascular disease (RVD), and RVD+Vitamins kidneys. Vitamin supplementation normalized the expression of pro-fibrotic TGF-$\beta$ and TIMP-1, and it improved MMP-2 and ubiquitin, suggesting a decrease pro-fibrotic activity accompanied by augmented extracellular and intracellular removal. *$P < 0.05$ *versus* normal, †$P < 0.05$ *versus* RVD. Magnification, ×40.

vascular, tubular, and glomerular injury. Many forms of renal disease are modulated by increased oxidative stress, and experimental blockade of the oxidative stress pathway with antioxidant vitamins in several disease models has been shown to decrease renal injury and glomerulosclerosis (23–25) and improve renal hemodynamics in models of Goldblatt hypertension (14,26). However, the potential of this approach to protect the stenotic kidney with RVD, which involves several concurrent renal insults (5) and may be more similar to the human disease, has not been explored.

Diet-induced HC is a well-accepted method to simulate early atherosclerosis (4,10,27). We have previously shown that HC resulted in renal functional impairment, associated with increased systemic oxidative stress, intrarenal inflammation, and vascular and tubulointerstitial injury (4,13,19) (Table 3). However, these changes are significantly accentuated in our experimental model of RVD, in which coexisting hypercholesterolemia and RAS synergistically activate deleterious mechanisms that amplify renal injury (4,5). This model exhibits impaired renal functional response to challenge and decreased fluid reabsorption due to early tubular injury. Furthermore, the stenotic kidney shows a marked increase in both systemic and renal oxidative stress, which has the potential to mediate renal dysfunction, inflammation, and fibrosis (4,14). Supporting this view, the current study shows the benefits from antioxidant intervention achieved in our RVD model. Because of the similar degree of stenosis, basal renal hemodynamics and function were similarly decreased in RVD and RVD+Vitamins compared with controls. Medullary perfusion remained similar among the groups, likely as a renal defense mechanism to preserve the medulla and reflecting the independent regulation of medullary blood flow from that of the cortex (28,29).

Renal hemodynamics and function remained attenuated in both RVD groups during the endothelium-independent challenge with SNP, possibly due to unabated increased activity of vasoconstrictors (*e.g.* endothelin-1) that the low systemic dose that we used of this NO donor might have been insufficient to negate. Nevertheless, diminished sensitivity to NO and smooth muscle cell dysfunction cannot be excluded either. Indeed, increased expression of eNOS, as observed in RVD+Vitamins, might have been necessary to augment renovascular responses to the endothelium-dependent vasodilator Ach in this group. Antioxidant vitamin intervention greatly improved RBF, GFR, and regional perfusion responses to Ach. This improvement in renal functional responses may have resulted from decreased abundance of the vasoconstrictor superoxide anion (suggested by the normalized expression of the NAD(P)H-oxidase subunits p67phox and p47phox), increased expression of eNOS, decreased peroxynitrite formation, and increased bioavailability of NO. Peroxynitrite and ROS may also increase the pro-inflammatory iNOS and NF-$\kappa$B (30,31), as observed in the RVD kidneys. Increased iNOS activity may further inhibit eNOS activity, thereby leading to renal vasoconstriction and reduction in GFR (32), and NF-$\kappa$B may increase transcription of genes, leading to inflammation and injury (31), especially in the sensitive tubules. Remarkably, stenotic kidneys treated with antioxidant vitamins showed decreased expression of both iNOS and NF-$\kappa$B, reflecting reduced intrarenal inflammation, which was further supported by the reduced presence of ED-$1^+$ and CD-$3^+$ cells. Since iNOS-derived tubular NO may also impair tubular function and decrease ITC, attenuated iNOS expression may account for the improvement in tubular function and GFR observed in vitamin-treated RVD (33).

Furthermore, the impairment in renal function in RVD due to increased oxidative stress is accompanied by augmented tubulointerstitial and glomerular fibrosis (4). The excessively produced ROS may mediate renal fibrotic injury through activation of the TGF-$\beta$ pathway to facilitate extracellular matrix (ECM) accumulation (34). Indeed, TGF-$\beta$ has been shown to increase various ECM proteins and TIMP while decreasing MMP (35), the major regulators of renal ECM degradation (36). In addition, ROS can directly diminish the activity of

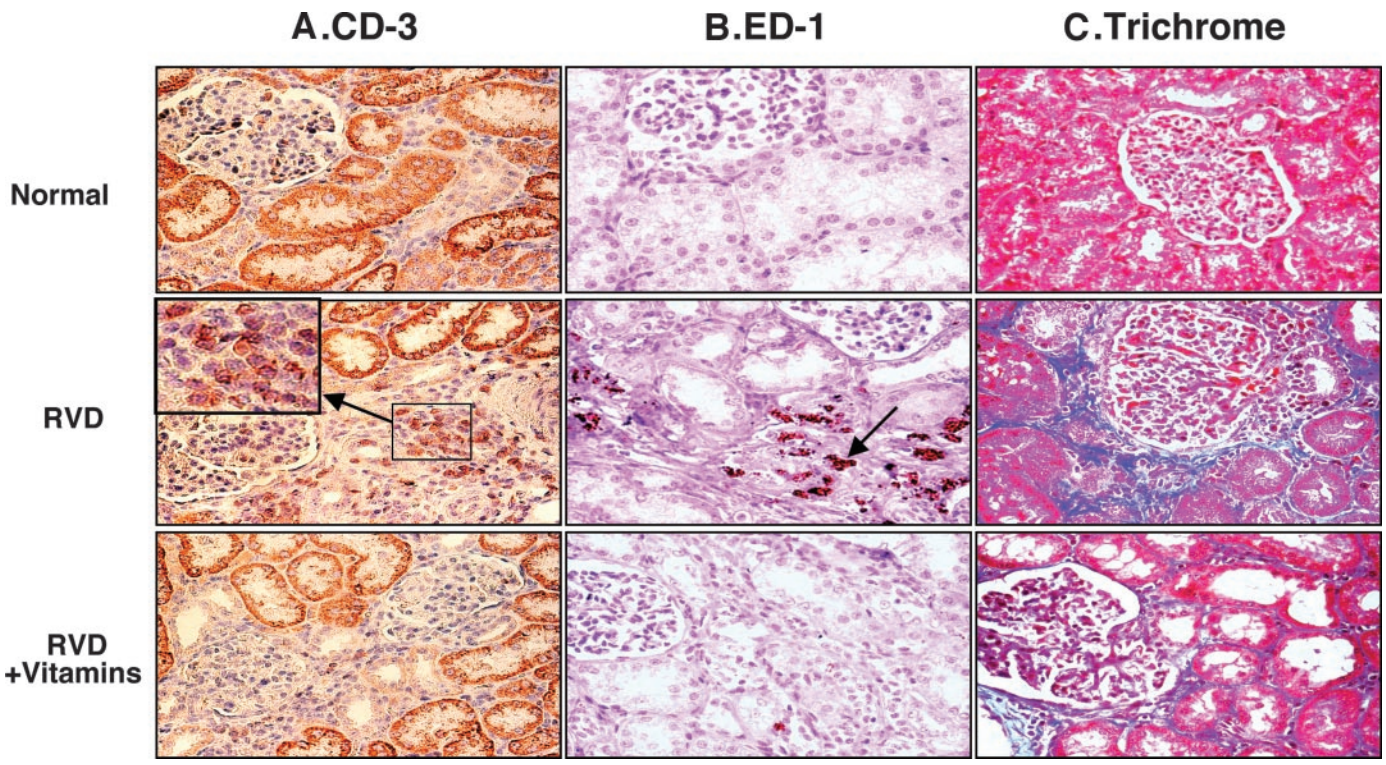


*Figure 5.* Representative stainning for CD-3 (A), ED-1 (B), and trichrome (C) in normal, renovascular disease (RVD), and RVD+Vitamins kidneys, showing a substantial decrease in inflammation, glomerulosclerosis, and tubulointerstitial fibrosis compared to RVD. Magnification, ×40.

MMP-2 (37), and oxidation of ECM can modulate its susceptibility to degradation, which may account for ECM accumulation and glomerulosclerosis (38). Moreover, augmented intrarenal oxidized LDL may elicit a fibrogenic response by leading to both an increase in intrarenal and extracellular matrix protein synthesis and decrease their degradation (39,40). We have previously shown that the significant increase in activities of extracellular and intracellular protein degradation systems, likely mediated by oxidative stress (41), was still relatively blunted in RVD and insufficient to afford adequate protein degradation and removal, resulting in substantial renal scarring (5). Accordingly, RVD animals treated with vitamins C and E showed ameliorated tubulointerstitial and glomerular fibrosis, attenuated TGF-$\beta$ and TIMP-1 expression, and augmented immunoreactivity of MMP-2. Notably, the expression of the ubiquitin/proteasome system was also increased at tubular and glomerular compartments in vitamin-treated RVD. We have previously shown that ubiquitin expression was increased in the coronary arteries in early atherosclerosis and decreased with antioxidant intervention (41). Since ubiquitin expression was further increased with antioxidant vitamins in the ischemic kidney of RVD, this may potentially reflect a tissue-specific and/or disease-specific effect of antioxidant intervention on regulation of the ubiquitin/proteasome system. This improvement in tissue remodeling may account for the slightly (albeit NS) lower renal volume (and consequently RBF and GFR) in RVD+Vitamins. Overall, these findings suggest that chronic blockade of multiple oxidative stress pathways in early RVD tends to restore the altered balance between protein degradation and removal responsible for renal scarring (5), and thereby to preserve the structure of the stenotic kidney.

Increased abundance of ROS may initially lead to renal microvascular endothelial dysfunction (42) similar to that observed in the myocardial microcirculation, which may precede and subsequently be aggravated by development of obstructive lesions in the main renal artery. Notably, although blockade of the oxidative stress pathway substantially attenuated renal injury, it did not completely abolish it, suggesting that additional pathophysiological mechanisms might be involved. The decrease in RBF and subsequent sustained activation of the renin-angiotensin system is likely a major trigger for renal injury in this model (43,44). Furthermore, increased oxidation of LDL in atherosclerosis may also induce structural modifications of cell proteins and impair cell viability and programmed cell death (39). Although vitamins C and E can protect LDL from oxidation (45,46), it is possible that this substantial increase in plasma LDL resulted in abundance of substrate that exceeded this protective mechanism. An effect of antioxidants such as vitamin C and E on BP is not consistently observed (23,47,48) and was not observed in our study, nor did it interfere with our results. PRA was similar among the groups, as common in chronic untreated, experimental renovascular hypertension (4,5,18). Notably, we have previously shown that PRA in our model shows a transient increase 4 to 5 wk after induction of a stenosis (from 0.4 ± 0.1 to 4.4 ± 1.9

*Table 3.* Qualitative evaluation of the systemic and renal effects of swine hypercholesterolemia (HC) alone or in conjunction with renal artery stenosis (RVD) compared to normal pigs[a]

| | HC | RVD |
|---|---|---|
| Systemic | | |
| total cholesterol (mmol/L) | ↑ | ↑ |
| mean arterial pressure (mmHg) | N | ↑ |
| plasma superoxide dismutase activity (U/mg) | ↓ | ⇓ |
| Renal | | |
| renal blood flow (ml/min) | N | ↓ |
| glomerular filtration rate (ml/min) | N | ↓ |
| perfusion (ml/min per cc) | | |
| cortex | N | ↓ |
| medulla | N | N |
| intratubular fluid concentration (arbitrary units) | | |
| proximal nephron | N | ↓ |
| distal nephron | N | ↓ |
| tissue superoxide dismutase activity (mU/mg) | N | ↓ |
| nitrotyrosine | ↑ | ⇑ |
| NF-$\kappa$B | ↑ | ⇑ |
| TGF-$\beta$ | ↑ | ⇑ |
| MMP-2 | N | ↑ |
| ubiquitin | N | ↑ |
| trichrome | ↑ | ⇑ |
| glomerular score | N | ⇑ |

[a] N, no difference from normal pigs.

ng/ml per hour) and lateralizes to the stenotic kidney but returns to baseline levels (to 0.4 ± 0.1 ng/ml per hour) by 10 to 12 wk (12). This may be partly due to the relatively moderate stenosis and the contralateral kidney, which suppresses renin release to somewhat counterbalance systemic PRA.

Our study involved a relatively early stage of this disease, and the chronic antioxidant intervention was initiated before instigation of renal injury. Clinical atherosclerotic RVD is a progressive disorder, and longer duration and chronicity likely eventuate in renal damage and irreversible scarring. Nevertheless, this study demonstrates that chronic antioxidant intervention with vitamins C and E in a model of early RVD improves RBF, GFR, and regional perfusion responses. Importantly, the stenotic RVD+Vitamins kidney showed attenuated intrarenal inflammation, fibrosis, glomerulosclerosis, and tissue remodeling. Thus, the current study supports a potential role for antioxidant vitamin intervention in preserving the kidneys in RVD and reveals a novel renoprotective effect of antioxidant intervention in the setting of RVD, which may potentially delay progression to ESRD.

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