Beneficial Effects of Antioxidant Vitamins on the Stenotic Kidney

Alejandro R. Chade, Martin Rodriguez-Porcel, Joerg Herrmann, James D. Krier, Xiangyang Zhu, Amir Lerman, Lilach O. Lerman

Abstract—Renal artery stenosis (RAS) may lead to renal injury, partly mediated through increased oxidative stress. However, the potential effects of chronic oral antioxidant intervention on the stenotic kidney remain unknown. This study was designed to test the hypothesis that chronic antioxidant vitamin supplementation in RAS would preserve renal function and structure. Single-kidney hemodynamics and function were quantified in vivo in pigs using electron-beam CT after 12 weeks of unilateral RAS (n=7), a similar degree of RAS orally supplemented with vitamins C (1 g) and E (100 IU/kg) (RAS+Vitamins, n=7), or controls (normal, n=7). Renal tissue was studied ex vivo using Western blotting and immunohistochemistry. Mean arterial pressure was similarly elevated in both RAS groups, while ischemic renal volume and glomerular filtration rate were similarly reduced. Renal blood flow was decreased in RAS compared with normal (326.5±99.9 versus 553.4±48.7 mL/min, respectively, P=0.01), but preserved in RAS+Vitamins (485.2±104.1 mL/min, P=0.3 versus normal). The marked increase in the expression of the NADPH-oxidase subunits p47phox and p67phox, nitrotyrosine, endothelial and inducible nitric oxide synthase, and nuclear factor-κB observed in RAS (P<0.05 versus normal) was normalized in RAS+Vitamins (P>0.1). Furthermore, trichrome staining and the expression of transforming growth factor-β and tissue inhibitor of matrix-metalloproteinase-1 were also decreased in RAS+Vitamins. In conclusion, chronic blockade of the oxidative stress pathway in RAS using antioxidant vitamins improved renal hemodynamics and decreased oxidative stress, inflammation, and fibrosis in the ischemic kidney. These observations underscore the involvement of oxidative stress in renal injury in RAS and support a role for antioxidant vitamins in preserving the ischemic kidney. (Hypertension. 2003;42:605-612.)

Key Words: kidney ■ hypertension, renovascular ■ regional blood flow ■ oxidative stress

Renal artery stenosis (RAS) is a major cause of renovascular hypertension and may lead to deterioration of renal function, renal tissue injury (ischemic nephropathy), and eventually end-stage renal disease (ESRD).1,2 In recent years it has become evident that one of the mechanisms by which prolonged renal hypoperfusion may progressively impair renal function and induce irreversible renal damage is increased oxidative stress.3,4 This may be due to sustained activation of the renin-angiotensin system in response to renal hypoperfusion, because angiotensin II (AII) is a potent stimulus for NAD(P)H oxidase-induced generation of reactive oxygen species (ROS), such as the superoxide anion.5,6 In turn, ROS avidly react with nitric oxide (NO) to produce the cytotoxic peroxynitrite (which can nitrate proteins and damage other molecules7) and elicit the formation of several other vasoactive, inflammatory, and growth-promoting factors in the kidney.

We have previously shown that the decrease in renal blood flow (RBF), glomerular filtration rate (GFR), and regional renal perfusion in experimental RAS was associated with intrarenal inflammation, fibrosis, and increased oxidative stress.3,8,9 Increased oxidative stress is involved in the mechanisms of many forms of renal injury, and evidence of the potential benefits of antioxidant intervention with vitamins in cardiovascular10,11 and renal disease12–14 is accumulating. However, the potentially beneficial effects of antioxidant vitamins on the stenotic kidney have not been explored. Thus, the present study was designed to test the hypothesis that blockade of the oxidative stress pathway in RAS, using chronic supplementation with antioxidant vitamins, would decrease renal damage.

Methods

The Institutional Animal Care and Use Committee approved all procedures. Twenty-one domestic pigs (55 to 65 kg) were studied after 12 weeks of observation. In 14 pigs, a local-irritant coil was placed in the left main renal artery at baseline, inducing gradual development of unilateral RAS, as previously described.3,9,15 Animals were then fed for 12 weeks with either a normal diet (RAS, n=7) or a normal diet orally supplemented with daily doses of vitamins C (1000 mg) and E (100 IU/kg; RAS+Vitamins, n=7). We
have previously shown that this regimen provided effective blockade of the oxidative pathway in the pig. The degree of RAS was subsequently measured by quantitative renal angiography. The remaining 7 pigs were fed with a normal diet and used as controls (normal, n=7).

**Measurements of Renal Hemodynamics and Function**

Evaluation of regional renal function distal to RAS was achieved in vivo by using electron-beam computed tomography (EBCT), an ultra-fast scanner, which provides accurate, reproducible, and non-invasive quantifications of single kidney volume, regional perfusion, blood flow, GFR, and segmental tubular function. On the day of the studies, each animal was anesthetized, and EBCT studies were then performed as previously detailed. Blood pressure was measured through a carotid arterial catheter, and systemic venous blood samples were collected for measurement of plasma renin activity (PRA, RIA) and serum creatinine (photometry).

After completion of the in vivo studies, the pigs were euthanized with a lethal intravenous injection of Sleepaway® (sodium pentobarbital, 100 mg/kg intravenous, Fort Dodge Laboratories, Inc). Kidneys were removed using a retroperitoneal incision, immediately frozen-in liquid nitrogen and stored at −80°C, or preserved in formalin. In vitro studies were then performed. Superoxide dismutase (SOD) activity was quantified in homogenized renal tissue using spectrophotometry. Renal morphology was examined in sections stained with trichrome. Protein expression of the NAD(P)H-oxidase subunits p47phox and p67phox, nitrotyrosine (as a footprint for peroxynitrite formation in vivo), endothelial nitric oxide-synthase (eNOS), the proinflammatory transcription factor nuclear factor-κB (NF-κB), and the progrowth factor tissue inhibitor of metalloproteinase (TIMP)-1 were measured by both Western blotting and immunohistochemistry (IHC). In addition, IHC for the proinflammatory isofrom inducible-NOS (iNOS) and expression of the profibrotic transforming-growth factor (TGF)-β were also investigated.

**Superoxide Dismutase Assay**

Total SOD activity was measured in renal tissue using a superoxide dismutase assay kit (R&D systems, Inc), following vendor instructions. Briefly, frozen renal tissue was pulverized and homogenized at 4°C in 1× specific SOD reaction buffer. The homogenized tissue was centrifuged for 15 minutes at 4°C at 14 000g and the supernatant placed on ice. The following components were then sequentially added and thoroughly mixed: distilled water, 25× reaction buffer, xanthine solution, and nitro blue tetrazolium (which absorbs light at 560 nm). The sample was then placed into a visible spectrophotometer at a wavelength of 560 nm, and the absorbance reading was set to zero. To start the reaction, xanthine oxidase solution was added to the preparation, and the absorbance was then read in each sample every 30 seconds for a period of 5 minutes. SOD presence was measured through a carotid arterial catheter, and systemic venous blood samples were collected for measurement of plasma renin activity (PRA, RIA) and serum creatinine (photometry).

**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-variable curve-fits, and the area enclosed under each segment of the curve and its first moment were calculated using the curve-fitting parameters. These were used to calculate single-kidney RBF, GFR, and intratubular fluid concentration (ITC), using previously validated methods. ITC indicates the concentration of the contrast medium in each tubular segment and serves as an index of tubular fluid reabsorption.

**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), as previously detailed. Glomerular score (percentage of sclerotic glomeruli) was assessed by recording the number of sclerotic glomeruli out of 100 counted glomeruli.

**Statistical Analysis**

Results are mean±SEM. Comparisons within groups were performed using a paired Student t test, and among groups using ANOVA, with the Bonferroni correction for multiple comparisons, followed by an unpaired Student t test. Statistical significance was accepted for P<0.05.

**Results**

The degree of stenosis was similar in stenotic kidneys from both RAS groups (Table 1). Mean arterial pressure (MAP) and plasma creatinine were significantly elevated in both RAS and RAS+Vitamins compared with normal. Systemic PRA was not different among the groups after 12 weeks (Table 1), as previously observed in this and other animal models of RAS.

**Renal Hemodynamics and Function**

Total renal volume was significantly and similarly reduced in both groups with RAS (Table 1). RBF and cortical perfusion were significantly reduced in RAS animals compared with normal. However, RAS supplemented with vitamins showed normalization of RBF and a significant increase in cortical perfusion compared with RAS and normal as well (Table 1). Medullary perfusion remained unchanged among the groups. Furthermore, renal vascular resistance was elevated only in RAS and was not different from normal in RAS+Vitamins. GFR was similarly decreased in both RAS groups compared with normal (Table 1). ITC was significantly increased in the proximal nephron of RAS compared with normal animals, suggesting augmented tubular fluid reabsorption, but tended to be lower in RAS+Vitamins (Table 1, P=0.07 versus RAS). The ITC in the loop of Henle was increased in RAS and similar to normal in RAS+Vitamins, whereas in the distal nephron it was not different among the groups.

**Immunohistochemistry**

Immunohistochemistry for eNOS (Transduction Laboratories; 1:500), iNOS (Affinity Bioreagents; 1:500), NF-κB (Santa Cruz Biotechnology Inc; 1:50), nitrotyrosine (Cayman; 1:20), TGF-β (Santa Cruz Biotechnology Inc; 1:10), and TIMP-1 (Santa Cruz Biotechnology Inc; 1:100) was performed on 5 μm-thick slices of either frozen (eNOS) or deparaffinized renal tissue. The secondary antibody, IgG Envision Plus (Dako), was followed by staining with the Vector NovaRED substrate kit (Vector Laboratories), and slides were counter-stained with hematoxylin.
Redox Status
SOD activity was significantly decreased in RAS kidneys compared with normal (30.5±1.4 versus 41.3±1.6 μU/mg protein, \( P=0.03 \)) and remained attenuated in RAS+Vitamins (20.3±2.2 μU/mg, \( P=0.01 \) versus normal). However, the increased protein expression of both the p47phox and p67phox subunits of NAD(P)H oxidase observed in RAS was normalized in RAS+Vitamins (Figures 1a and 1b), suggesting decreased potential for generation of superoxide. In addition, tubular and glomerular protein expression of nitrotyrosine was significantly elevated in RAS kidneys compared with normal, but dramatically reduced in RAS+Vitamins (Table 2, Figure 1c, \( P<0.03 \) versus RAS), implying decreased production of the cytotoxic peroxynitrite. Protein expression of eNOS, which was significantly increased in renal endothelial cells of RAS animals (\( P<0.02 \) versus normal, Figure 2a), was normalized in RAS+Vitamins (\( P=0.37 \) versus normal, \( P<0.02 \) versus RAS).

Renal Inflammation and Fibrosis
Tubular (mainly proximal) and glomerular expression of the proinflammatory factors iNOS and NF-kB, which were markedly elevated in RAS animals, was substantially decreased in the vitamin-treated group (Table 2, Figures 2b and 2c, respectively), indicating a decrease in renal inflammation. Furthermore, the expression of the growth factor TGF-β was increased in the tubular and glomerular compartments of RAS, accompanied by a marked increase in perivascular, glomerular, and tubulointerstitial fibrosis. In contrast, kidneys from animals treated with vitamins showed a significant decrease in TGF-β expression (Figure 3a), although the glomerular score (representing the degree of glomerulosclerosis) was unaltered (Table 2). Nevertheless, RAS+Vitamins exhibited a tendency for somewhat lower trichrome staining in the tubular and interstitial compartments (\( P=0.09 \) versus RAS), suggesting a decrease in renal fibrosis as a result of chronic antioxidant supplementation (Table 2, Figure 3b). This was associated with attenuated expression of TIMP-1 (\( P<0.01 \)) in RAS+Vitamins, compared with the markedly elevated expression observed in untreated-RAS animals (Table 2, Figure 3c), implying an overall attenuation of the scarring process in vitamin-supplemented animals.

Discussion
This study demonstrates that chronic blockade of the oxidative stress pathway in renal artery stenosis using oral antioxidant vitamin supplementation improves renal hemodynamics and decreases oxidative stress, intrarenal inflammation, and tubulointerstitial fibrosis in the stenotic kidney. These results underscore the role of increased oxidative stress in the pathogenesis of ischemic nephropathy and suggest a role for antioxidant vitamins in preserving the function and structure of the stenotic kidney.

RAS may lead to hypertension and progressive renal failure, and in experimental models of RAS the contribution of increased oxidative stress to the pathogenesis of renal injury has been demonstrated. Indeed, renal hypoperfusion disrupts the balance among intrarenal vasoactive factors that regulate vascular tone and tissue growth and leads to increased generation of ROS. ROS may inhibit NO-mediated vasodilatation, modulate cell growth and proliferation, and accelerate renal inflammation and fibrosis. Therefore, blockade of the oxidative stress pathway might conceivably improve the hemodynamics and function of the stenotic kidney.

### Table 1. Systemic Characteristics and Single-Kidney Hemodynamics in Normal, RAS, and RAS+Vitamins Pigs

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal (n=7)</th>
<th>RAS (n=7)</th>
<th>RAS+Vitamins (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degree of stenosis, %</td>
<td>...</td>
<td>74.5±7.1*</td>
<td>68.1±3.5*</td>
</tr>
<tr>
<td>Mean arterial pressure, mm Hg</td>
<td>99.8±4.5</td>
<td>122.8±7.9*</td>
<td>126.3±4.3*</td>
</tr>
<tr>
<td>Plasma renin activity, ng/mL/h</td>
<td>0.39±0.06</td>
<td>0.31±0.1</td>
<td>0.32±0.1</td>
</tr>
<tr>
<td>Creatinine, μmol/L</td>
<td>127.9±6.0</td>
<td>167.9±11.1*</td>
<td>176.8±17.7*</td>
</tr>
<tr>
<td>Kidney volume, cc</td>
<td>143.8±8.8</td>
<td>89.2±21.3*</td>
<td>89.1±17.5*</td>
</tr>
<tr>
<td>Renal blood flow, mL/min</td>
<td>554.4±46.3</td>
<td>328.5±59.9*</td>
<td>485.2±104.1</td>
</tr>
<tr>
<td>Renal vascular resistance</td>
<td>0.18±0.09</td>
<td>0.38±0.08*</td>
<td>0.26±0.04</td>
</tr>
<tr>
<td>Perfusion, mL/min/cc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortex</td>
<td>4.2±0.3</td>
<td>3.0±0.6</td>
<td>6.3±0.4†</td>
</tr>
<tr>
<td>Medulla</td>
<td>2.6±0.3</td>
<td>2.7±0.2</td>
<td>3.1±0.6</td>
</tr>
<tr>
<td>Glomerular filtration rate, mL/min</td>
<td>69.3±4.4</td>
<td>39.8±11.2*</td>
<td>24.2±27.2*</td>
</tr>
<tr>
<td>Intratubular fluid concentration, AU</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proximal tubule</td>
<td>3.8±0.2</td>
<td>8.3±1.2*</td>
<td>6.1±0.5‡</td>
</tr>
<tr>
<td>Henle’s loop</td>
<td>8.2±1.3</td>
<td>14.1±1.6*</td>
<td>9.5±1.4</td>
</tr>
<tr>
<td>Distal tubule</td>
<td>6.9±0.9</td>
<td>7.8±1.6</td>
<td>5.8±0.5</td>
</tr>
<tr>
<td>Collecting duct</td>
<td>11.3±1.4</td>
<td>9.9±2.2</td>
<td>12.3±1.4</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SEM. RAS indicates renal artery stenosis; RAS+Vitamins, RAS pigs treated with vitamins.

* \( P<0.05 \) vs normal; † \( P<0.05 \) vs RAS; ‡ \( P=0.07 \) vs RAS.
We have previously shown in a pig model that RAS induces a significant reduction in RBF and GFR and an increase in tubular fluid reabsorption in the proximal nephron (which, using our method, extends to the Loop of Henle), consistent with the main site of action of angiotensin II. These results were also associated with increased oxidative stress, decreased scavenging activity, and inflammation. The current study extends our previous observations and shows that these changes in RAS were accompanied by increased protein expression of the NAD(P)H-oxidase subunits p47phox and p67phox. The NAD(P)H-oxidase is the major source of vascular and tissue superoxide anion, and both p47phox and p67phox are cytosolic subunits of the enzyme isoform located in vascular smooth muscle, endothelial, mesangial, and adventitial cells. Recent studies have reinforced the mandatory role of p47phox phosphorylation for activation of the NAD(P)H oxidase enzyme, especially via angiotensin II–mediated mechanisms. The increased expression of nitrotyrosine also supports increased abundance of superoxide in the renal tissue. In addition, we observed increased cortical expression of eNOS in RAS, as previously demonstrated in the ischemic kidney. This increase might reflect a compensatory mechanism triggered by an increase in renovascular resistance and, consequently, shear stress, and aimed at sustaining renal perfusion via production of nitric oxide. Increased production of hydrogen peroxide may also upregulate eNOS, although this mechanism may be less likely in our study, considering the low activity of SOD in the

TABLE 2. Morphological Evaluation and Immunostaining in Normal, RAS, and RAS + Vitamins Pigs

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Normal (n=7)</th>
<th>RAS (n=7)</th>
<th>RAS + Vitamins (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrotyrosine</td>
<td>7.3±0.4</td>
<td>9.4±0.3*</td>
<td>1.5±0.2†</td>
</tr>
<tr>
<td>eNOS</td>
<td>2.8±0.3</td>
<td>6.7±0.4*</td>
<td>3.0±0.4†</td>
</tr>
<tr>
<td>iNOS</td>
<td>3.2±0.4</td>
<td>9.9±0.4*</td>
<td>3.9±0.2†</td>
</tr>
<tr>
<td>NF-κB</td>
<td>0.3±0.04</td>
<td>2.7±0.9*</td>
<td>0.2±0.04†</td>
</tr>
<tr>
<td>TGF-β</td>
<td>1.5±0.09</td>
<td>6.6±0.6*</td>
<td>1.4±0.1†</td>
</tr>
<tr>
<td>Trichrome</td>
<td>3.3±0.2</td>
<td>6.5±0.3*</td>
<td>5.4±0.6‡</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>1.9±0.1</td>
<td>3.3±0.1*</td>
<td>1.6±0.2†</td>
</tr>
<tr>
<td>Glomerular score</td>
<td>0.0±0.0</td>
<td>4.2±0.5*</td>
<td>3.9±1.3*</td>
</tr>
</tbody>
</table>

Data are expressed as percentages of renal area; mean±SEM.
*P<0.05 vs normal; †P<0.05 vs RAS; ‡P=0.09 vs RAS.
RAS group. Alternatively, the increased protein expression may reflect uncoupled eNOS, which may in fact generate ROS and consequently further increase oxidative stress.

Indeed, increased oxidative stress in acute ischemic renal failure contributes to renal injury, which can be mitigated with acute antioxidant intervention. Notably, the current study, using chronic oral antioxidant intervention, showed increased RBF and improvement in tubular function in the RAS+Vitamins group. These were attended by a dramatic attenuation in NAD(P)H-p47phox, -p67phox, and nitrotyrosine expression, which might reflect reduced production of the renal vasoconstrictor superoxide and the cytotoxic peroxynitrite. The decreased formation of peroxynitrite conceivably accounted for the decreased expression of iNOS and NF-κB and thereby decreased inflammation in RAS+Vitamins. The decreased formation of the vasoconstrictor superoxide might have served and sufficed to increase RBF, because eNOS expression was in fact normalized following antioxidant supplementation. Although antioxidant vitamins may directly upregulate eNOS, the compensatory increase in eNOS in the ischemic kidney might have no longer been necessary in the face of decreased oxidative stress. The decrease in eNOS expression might have been mediated by attenuated superoxide and consequently decreased hydrogen peroxide formation as a result of antioxidant intervention. Hence, the decrease in renovascular resistance observed in RAS+Vitamins might have decreased shear stress in the stenotic kidney (and consequently eNOS expression) and thereby decreased inflammation in RAS+Vitamins. The effective increase in RBF accompanied by unaltered GFR in RAS+Vitamins may implicate efferent vasodilation in these effects and suggests a decrease in filtration fraction, which might have contributed.
to the remaining elevation in proximal fluid reabsorption. In addition, renal tissue SOD activity was not normalized in RAS+Vitamins, which might be related to direct downregulation of SOD by chronic exposure to high levels of vitamin E.39 However, because superoxide formation was likely significantly decreased in RAS+Vitamins, the diminished scavenging activity might be less functionally meaningful in these kidneys.

ROS mediate several pathogenic processes that affect cell proliferation and differentiation and may increase renal fibrosis, partly via activation of TGF-β, a potent fibrogenic renal growth factor. We have previously shown that RAS kidneys showed increased expression of TGF-β and intrarenal fibrosis.3,9 Moreover, TGF-β has been shown to increase deposition of extracellular matrix proteins and upregulate TIMPs, providing the optimal environment for progressive matrix accumulation.40 Indeed, we observed that the increased TGF-β expression in RAS kidneys was accompanied by increased immunoreactivity of TIMP-1, suggesting augmented tissue remodeling in this group. However, supplementation with vitamins C and E resulted in TGF-β and TIMP-1 expression that was not different from normal kidneys. Interestingly, the degree of glomerulosclerosis in RAS kidneys was unaffected by antioxidant intervention, suggesting a role for additional progrowth factors not modulated by ROS. Notably, the glomerular score at this early stage was rather small even in RAS, and antioxidant vitamins may still play a role in delaying progression of renal injury at a later phase. Moreover, tubulointerstitial fibrosis was blunted in RAS+Vitamins compared with RAS alone, indicating that chronic antioxidant supplementation may indeed attenuate the scarring process in the early stages of this disease.

The augmented oxidative stress contributes to the enhanced basal vascular tone and maintenance of elevated blood pressure in RAS, as observed in animal5,8 and human41 studies. However, although the current study showed that oxidative stress blockade with antioxidant vitamins resulted in a significant improvement in renovascular function, this was dissociated from a decrease in blood pressure. Importantly, Welch et al4 have recently shown a significant decrease in blood pressure in renovascular hypertensive rats after a 2-week infusion of the radical scavenger Tempol, but the effects of antioxidant vitamins on blood pressure are

Figure 3. Representative renal immunoblots and immunohistochemistry demonstrating protein expression of transforming-growth-factor (TGF)-β (a) and tissue-inhibitor of metalloproteinases (TIMP)-1 (c) and staining for trichrome (b) in normal, renal artery stenosis (RAS), and RAS+Vitamins. Vitamin-treated RAS kidneys showed a substantial attenuated expression of these profibrotic factors and a decrease in renal fibrosis compared with RAS. *P<0.05 versus normal. Magnification: ×40.
inconsistent.\textsuperscript{12,23} It is possible that beneficial chronic effects of antioxidant vitamins are derived more from decreases in lipid peroxidation and tissue injury\textsuperscript{16} than from regulation of blood pressure.

**Perspectives**

This study demonstrates that blockade of the oxidative stress pathway in a model of unilateral RAS augmented RBF and cortical perfusion, improved tubular function, and ameliorated intrarenal inflammation and tubular and interstitial fibrosis. These observations support a renoprotective role for antioxidant vitamins C and E on the stenotic kidney and may indicate their potential for attenuating renal functional and structural alterations in this disease. Alas, these results will need to be confirmed in clinical studies. In addition, the beneficial effects of chronic blockade of the oxidative stress pathway in RAS might conceivably be extended to the contralateral kidney as well, although these would need to be assessed in future studies. Moreover, preservation of renal function and thwarting glomerulosclerosis may require additional strategies to prevent the advent and progression of renal compromise.

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**References**


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