Antioxidant-Rich Diet Relieves Hypertension and Reduces Renal Immune Infiltration in Spontaneously Hypertensive Rats

Bernardo Rodriguez-Iturbe, Chang-De Zhan, Yasmir Quiroz, Ram K. Sindhu, Nosratola D. Vaziri

Abstract—Previous studies have demonstrated that oxidative stress contributes to hypertension and treatments with either antioxidant or immunosuppressive/anti-inflammatory agents improve hypertension in spontaneously hypertensive rats (SHR). The present study was performed to determine if the antihypertensive effects of an antioxidant-rich diet are associated with reduction in the renal immune infiltration. Rats were divided into experimental groups (n=5 each) that were followed 7 months after birth, during which they were fed either a regular or antioxidant-enriched (test) diet as follows: SHR-R group=regular diet; SHR-T group=test diet throughout the experiment; SHR-S group=test diet for 4 months switched to regular diet thereafter; WKY group=control rats given regular diet. The SHR-T rats showed a significant reduction in systolic blood pressure (mm Hg): SHR-T=179.6±12.9 versus SHR-R=207.5±9.6 (P<0.001) and plasma hydrogen peroxide concentration (SHR-T=15±4 μmol/L versus 34±9 in SHR-R rats). This was accompanied by significant reductions of renal tissue nitrotyrosine abundance, tubulointerstitial infiltration (cells/mm²) of lymphocytes (SHR-T=18±3 versus SHR-R=30±4, P<0.001), macrophages (SHR-T=17±3 versus SHR-R=22±3), and angiotensin II–positive cells (SHR-T=17±2 versus SHR-R=25±5, P<0.01). Results in the SHR-S group were intermediate between the SHR-R and SHR-T groups. The intensity of the infiltration of lymphocytes, macrophages, and angiotensin II–positive cells significantly correlated with systolic blood pressure. Thus, the present study demonstrates that an antioxidant-enriched diet reduces the renal interstitial inflammation and improves hypertension in SHR. These findings point to interrelation between oxidative stress and inflammatory reactivity in the pathogenesis of hypertension. (Hypertension. 2003;41:341-346.)

Key Words: antioxidants ■ lymphocytes ■ macrophages ■ immune systems ■ kidney ■ rats, spontaneously hypertensive

Evidence supporting the role of oxidative stress in the pathogenesis of hypertension has been advanced in recent years.1–4 The most compelling studies demonstrate that hypertension may, in part, develop as a result of increased reactive oxygen species5–11 and that a variety of antioxidant therapies ameliorate hypertension in rats with genetic and acquired forms of hypertension.12–21 Hypertensive effects of oxidative stress are the consequence, at least in part, of endothelial dysfunction resulting from disturbances of vasodilator systems, particularly degradation of nitric oxide by oxygen free radicals.24–26 In addition, oxidative stress in the kidney may be involved in the pathogenesis of salt retention. We have postulated that renal infiltration of immunocompetent cells may play a role in the pathogenesis of salt-sensitive hypertension.27,28 Tubulointerstitial infiltration of lymphocytes and macrophages is associated with the generation of reactive oxygen species (ROS) and angiotensin II–producing cells in experimental models of hypertension.29–32 As a consequence, increased sodium reabsorption, impaired pressure natriuresis, and decreased filtered sodium caused by glomerular vasoconstriction may develop,33 imposing a pathophysiological state that favors sodium retention (reviewed by Johnson et al34). Previous studies from our group have shown that reduction of the immune infiltration with an immunosuppressive drug, mycophenolate mofetil, improves oxidative stress as well as hypertension.29–32 The present investigations examine the opposite side of the same issue: whether the long-term administration of an antioxidant-rich diet would reduce the renal immune infiltration as well as improve systemic hypertension. Our findings indicate that an antioxidant-rich diet ameliorates hypertension in spontaneously hypertensive rats (SHR) in association with reduction in the infiltration of lympho-
Vitamin E, Vitamin C, Zinc, and Selenium Contents of Regular and Test Diets*

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<th>Content</th>
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<td>Ascorbic acid, ppm</td>
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*Both diets were purchased from Purina Mills Inc (Richmond, Ind). Regular diet is Rodent Laboratory diet #5001 and Test diet is diet #45328.

cytess and macrophages in tubulointerstitial areas of the kidney.

Methods

Animals and Experimental Design

Experiments were done in SHR and control Wistar-Kyoto rats (WKY) obtained from Charles River Laboratories. One-week-pregnant rats and their offspring were fed either a regular rodent laboratory diet or a test diet prepared by adding to the regular diet vitamin E, vitamin C, zinc, and selenium. Diets were purchased from Purina Mills Inc. The differences in the composition of the regular diet and the test diet are shown in the Table. The male offspring were observed for 7 months.

Animals were housed in a temperature-controlled light-regulated space with 12-hour light and dark cycles and were given unrestricted access to food and water throughout the experiments. The protocol used in this study was approved by the animal care and use committee of the University of California, Irvine.

The pregnant animals and their offspring were randomly assigned to the following experimental groups (n=5 each): SHR-R group, consisting of SHR rats that were given regular diet throughout the experiment (7 months); SHR-T group, consisting of SHR rats that were given test (antioxidant-rich) diet throughout the experiment; SHR-S group, consisting on rats that received test diet for 4 months and then were switched to a regular diet for the remaining 3 months; and the WKY group, consisting of WKY rats given a regular diet throughout the experiments.

At the end of the experiments, animals were euthanized after being anesthetized with intraperitoneal injections of pentobarbital sodium (Nembutal, 50 mg/kg). Blood was obtained and kidneys were removed. One kidney was used for histological and immunohistochemical studies and the other kidney was used to determine malondialdehyde (MDA) content. Serum and urine creatinine was determined by a kit purchased from Sigma Chemical Inc. Urinary protein and creatinine concentrations were determined in the 24-hour urine collections.

Plasma Hydrogen Peroxide

Plasma H₂O₂ concentration was determined by the quantitative H₂O₂ assay kit (OXIS International Inc). Nitryrosine abundance in renal tissue was determined by Western blot analysis as described in our earlier studies.³⁹

Blood Pressure Determinations

Blood pressure was determined by tail-cuff plethysmography, as described previously.³⁵ Conscious rats were placed on a heated pad in a temperature-controlled quiet room. After 15 minutes of rest with the tail placed inside a tail cuff, the cuff was inflated 3 to 4 times to condition the animal to the procedure; 4 consecutive measurements were taken and recorded (Harvard Apparatus Inc).

Histology

Coronal 3- to 4-μm sections of paraffin-embedded tissue fixed in 10% buffered formalin (American Master®Tech Scientific, Inc) were used for histological studies. Periodic acid-Schiff (PAS), trichromic, and hematoxylin and eosin staining were used to evaluate light microscopic findings. The entire cortical and juxtamedullary regions were examined. Glomerulosclerosis was defined as PAS-stained material without cellular elements with or without adhesion to Bowman’s capsule. Glomerulosclerosis was graded by the score described by Raji³⁶ and detailed in previous communications.³⁰ Tubulointerstitial damage was classified according to the extent (%) of areas of tubular damage by a scale ranging from 0 to 5+, described previously.³⁰–³²: 0=no changes present; 1+=<10%; 2+=10% to 25%; 3+=25% to 50%; 4+=50% to 75%; 5+=75% to 100%. All histological studies were done in a blinded manner.

Cellular Infiltration

Lymphocytes (CD5-positive cells) and macrophages (ED1-positive cells) were identified by avidin-biotin-peroxidase methodology. Angiotensin II-positive cells were identified by indirect immunofluorescence. Cellular counts within the glomeruli are given as positive cells per glomerular cross section (gcs) and in tubulointerstitial areas as positive cells per millimeter².

Antisera

Lymphocytes were identified with anti-CD5 monoclonal antibody (clone MRCOX19, Biosource) and macrophages with anti-ED1 monoclonal antibody (Harlan Bioproducts). Angiotensin II-producing cells were investigated with rabbit anti-human angiotensin II antisera (Peninsula Laboratories) with cross-reactivity to rat angiotensin II.²⁹–³² Secondary rat anti-mouse and donkey anti-rabbit antibodies with minimal cross-reactivity to rat serum proteins were obtained from Accurate Chemical and Scientific Co.

Renal MDA

Renal MDA content was determined in kidney homogenates by the method of Buege and Aust.³⁷

Statistical Calculations

Comparisons between groups were done by using multiple-group ANOVA analysis. Significant differences were evaluated with Tukey-Kramer posttests. Associations between variables were explored with linear (Pearson) as well as nonparametric (Spearman) correlation coefficients. Differences were considered significant when 2-tailed tests indicated values of P<0.05. Calculations were made with the help of a commercially available statistical package (Instat, GraphPad). Results are expressed as mean±SD throughout the article.

Results

Weight, Renal Function, Blood Pressure, and Indicators of Oxidative Stress

There were no significant differences in weight among the experimental groups and control animals. At the end of the experiment, the weights were as follows: WKY = 341±27.4 g; SHR-R group = 345±21.3 g; SHR-S group = 322±33.9 g; SHR-T group = 327±18.9 g.

Creatinine clearance (mL/min) at the end of the experiment was similar in the WKY control rats (1.23±0.27) and in rats from the SHR-R (1.03±0.2), SHR-S (1.11±0.22) and SHR-T group (1.25±0.19). Urinary protein excretion (mg/24 hours) was mildly but significantly (P<0.05) increased in all SHR groups as compared with the WKY group (SHR-R group = 38.6±6.2; SHR-S group = 36.9±7.4; SHR-T group = 39.9±6.3; WKY group = 26.9±4.9).
Mean renal MDA content (nmol/mg kidney protein) was higher in the SHR-R group (2.45±0.57) than in rats of the SHR-S group (2.25±0.37), SHR-T group (2.0±0.13), and the WKY group (2.16±0.10), but the differences were not statistically significant.

Plasma hydrogen peroxide concentration (μmol/L) in the SHR-R group (34±9) was significantly (P<0.05, ANOVA) higher than those found in the SHR-T group (15±4) and WKY (18±8) groups. The SHR-S group showed an intermediate plasma hydrogen peroxide level (24±4). Renal tissue nitrotyrosine burden (an indicator of NO inactivation by ROS) was significantly increased in the SHR-R group (1977±81) compared with the WKY group (1693±81, P<0.05). It was significantly lowered by antioxidant therapy in the SHR-T group (1611±80) and was raised slightly by switching to the regular diet in the SHR-S group (1649±86). (Data represent mean±SD of relative optical densities of bands on Western analysis.) These findings point to increased ROS in the untreated SHR and the efficacy of the antioxidant diet used.

As expected, SHR of all experimental groups were hypertensive (Figure 1). Rats from the SHR-T group that received antioxidant-rich diet throughout the experiment had a significant (P<0.01) reduction of blood pressure compared with rats of the SHR-R group that received a regular diet. (SHR-T=179.6±12.9 mm Hg versus SHR-R=207.5±9.6). The SHR-S group, in which the test diet was switched to a regular diet after 4 months (SHR-S, shaded bar), had intermediate blood pressure values (198±14.8 mm Hg).

Cellular Infiltration
The glomeruli did not present significant infiltration of lymphocytes and macrophages. Intraglomerular lymphocytes (CD5-positive cells/gcs) were 0.21±0.15 in WKY rats and 0.25±0.29, 0.3±0.45, and 0.1±0.22 in SHR-R, SHR-S, and SHR-T, respectively. Macrophages (ED1-positive cells/gcs) were also similar in all groups (WKY=0.4±0.42, SHR-R=0.38±0.48, SHR-S=0.30±0.27, SHR-T=0.20±0.27).

Figure 1. Systolic blood pressure at the end of the experimental period. All experimental groups had significantly (P<0.001) higher values than control WKY rats (open bar). Antioxidant-rich test diet reduced significantly (**P<0.01) the blood pressure in rats receiving the test diet (SHR-T, striped bar) with respect to rats on a regular diet (SHR-R, closed bar). Rats that were switched from test diet to regular diet (SHR-S, shaded bar) had intermediate degree of hypertension.

In contrast, there were significant differences in the tubulointerstitial infiltration of lymphocytes and macrophages in the experimental groups. SHR rats from all groups had significantly higher numbers of CD5-positive cells than WKY rats. As shown in Figure 2, the more intense infiltration of CD5-positive cells was observed in rats kept on the regular diet (SHR-R) and the lowest number of CD5-positive cells in rats kept on the antioxidant-rich diet (SHR-T). Rats that were switched from the test diet to a regular diet (SHR-S) had intermediate numbers (Figure 2).

Macrophage infiltration showed the same pattern, with the SHR-R group showing the highest number of ED1-positive cells. Rats switched from regular to antioxidant diet (SHR-S) had intermediate macrophage infiltration, and rats kept on the test diet (SHR-T) had the lowest number of tubulointerstitial macrophages (Figure 3).

Similar findings were demonstrated with respect to angiotensin II-positive cells. As shown in Figure 4, antioxidant diet reduced the number of angiotensin II-producing cells. This effect was more pronounced in rats that were kept on the test diet (Figure 4).

Figure 2. Tubulointerstitial infiltration of lymphocytes (CD5-positive cells) in 7-month-old SHR rats kept from birth on a regular diet (SHR-R, closed bar), rats switched from an antioxidant diet after 4 months (SHR-S, shaded bar), and rats kept on an antioxidant-rich test diet throughout (SHR-T, striped bar). *P<0.05, **P<0.001. Error bars are SD.

Figure 3. Tubulointerstitial infiltration of macrophages (ED1-positive cells) in 7-month-old SHR rats kept from birth on a regular diet (SHR-R, closed bar), rats switched from an antioxidant diet after 4 months (SHR-S, shaded bar), and rats kept on an antioxidant-rich test diet throughout (SHR-T, striped bar). *P<0.05, **P<0.001. Error bars are SD.
Histology

Light microscopy was essentially unremarkable in all groups of rats. Glomeruli were normal, with focal areas of increased cellularity. Tubulointerstitial areas showed focal areas of dilation, but trichromic and PAS staining did not show areas of fibrosis. Tubulointerstitial injury scores were similar in all experimental groups SHR-R 1.8 0.45; SHR-S 1.6 0.55; SHR-T 1.8 0.55; WKY 1.6 0.55). Representative photomicrographs showing light microscopy and immune histology are shown in Figure 5.

There were significant correlations between the systolic blood pressure levels and lymphocyte infiltration ($r$ 0.80, $P$ 0.0001), macrophage infiltration ($r$ 0.877, $P$ 0.0001), and angiotensin II–positive cells ($r$ 0.828, $P$ 0.0001). The correlations between SBP levels and the immune cell infiltration are shown in Figure 6.

Discussion

Long-term consumption of the antioxidant-fortified diet beginning in the prenatal period improved hypertension and ameliorated oxidative stress, as evidenced by the reductions of tissue nitrotyrosine and MDA contents as well as plasma hydrogen peroxide concentration in the SHR-T group. These findings are consistent with the results of the short-term studies carried by our group using the potent antioxidant lazoroid compound$^{22}$ as well as those carried out by Schnackenberg et al$^{14}$ using the superoxide dismutase mimetic agent tempol in this model. Together, these studies have provided compelling evidence for the role of oxidative stress in the pathogenesis of hypertension in SHR. Similarly, the presence of oxidative stress and its role in elevation of arterial pressure has been shown in various other forms of genetic and acquired hypertension including that seen with lead exposure,$^{9}$ chronic renal insufficiency,$^{10}$ experimental syndrome X,$^{38}$ salt sensitivity,$^{39}$ coarctation of the aorta,$^{5}$ angiotensin infusion,$^{40}$ preeclampsia,$^{41}$ and renal artery stenosis.$^{42}$

In the present study, we used an antioxidant cocktail that included vitamin E, vitamin C, selenium, and zinc. Earlier studies have documented the beneficial effects of vitamin E and vitamin C in ameliorating hypertension in hypertensive animals$^{9,10,22}$ and improving endothelial function in hyperten-

Figure 5. A. Glomeruli with partial collapse of the glomerular tuft and focal hypercellularity surrounded by tubules, some of which are dilated and with partial loss of epithelial cells but preserved basement membrane. Lymphocytes (CD5-positive cells) and macrophages (ED1-positive cells) infiltrating tubulointerstitial areas are shown in avidin-biotin peroxidase staining and FITC staining, respectively, in B and C. Angiotensin II–positive tubular cells and interstitial cells (peroxidase staining) are shown in D.
The observed effects are in agreement with our previous studies in SHR showing that the reduction in immune cell infiltration of the kidney by administration of an immunosuppressive anti-inflammatory drug results in amelioration of hypertension, which is coupled with a decline in renal malondialdehyde content and the number of superoxide-positive cells in the kidney. As emphasized in a recent review, oxidative stress may be a cause as well as a consequence of hypertension, and if present in the kidney, it may fuel a vicious cycle that includes renal inflammation and sodium retention. Although the available data do not allow definitive conclusion as to the primary or secondary nature of the inflammatory process in the pathogenesis of hypertension, the data provide convincing evidence for its role in the maintenance of hypertension.

Oxidative stress has been shown to raise arterial blood pressure by promoting functional nitric oxide deficiency (through NO inactivation and tetrahydrobiopterin depletion) and by augmenting arachidonic acid oxidation and formation of vasoconstrictive prostaglandin F2α. It is therefore not surprising that amelioration of oxidative stress with antioxidant therapy should improve hypertension. However, the mechanism responsible for the antioxidant therapy–induced amelioration of the inflammatory infiltration of the renal tissue shown here is less clear. It should be noted that reactive oxygen species have been shown to activate nuclear factor-κB, which can in turn promote transcription of genes encoding proinflammatory cytokines. This phenomenon can potentially explain the prevention of the inflammatory infiltration of the kidney in the antioxidant-treated SHR-T group. It is of interest that long-term control of hypertension with AT-1 receptor blocker losartan has been recently shown to prevent structural and functional deterioration of kidney in SHR. However, by attenuating the angiotensin-mediated upregulation of NAD(P)H oxidase, AT-1 receptor blockade exerts an antioxidant action that contributes to its antihypertensive and renal protective properties.

The correlation between the intensity of cellular infiltration and the severity of hypertension in the present study raises the possibility of a cause-effect relation that needs to be explored further. In addition, the present studies confirm that dietary antioxidant treatment improves hypertension in SHR and suggest that this therapeutic approach may be of clinical use.

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**References**


**Perspectives**

The central findings of this investigation are, first, that SHR consuming a regular diet have renal infiltration of lymphocytes, macrophages, and angiotensin II–positive cells in association with severe hypertension, and second, that an antioxidant-fortified diet reduces renal immune infiltration and ameliorates hypertension.


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