Interactions between oxidative stress and inflammation in salt-sensitive hypertension

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Our laboratory has shown that the Dahl S rat, when challenged with a high-salt diet, experiences both oxidative stress and inflammation (45), and this is associated with hypertension and considerable renal damage. The high-Na Dahl S rats also had marked increases in renal monocytes/macrophages with significant increases in the renal nuclear transcription factor NF-κB and decreases in renal plasma flow and glomerular filtration rate (GFR) (45). However, it is not clear whether the increases in oxidative stress in Dahl S rats on a high-salt diet help to stimulate renal NF-κB and renal proinflammatory cytokines and chemokines. The present study was designed to determine whether antioxidant treatment of Dahl S rats on high Na intake will prevent increases in renal oxidative stress and thus decrease renal inflammation and arterial pressure and the associated renal damage and dysfunction. A second goal was to determine whether the inflammatory response to a high Na intake in Dahl rats occurs only if the blood pressure increases. These goals were met by measuring renal levels of oxidative stress, NF-κB, interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), monocyte chemoattractant protein-1 (MCP-1), renal monocytes and macrophages, microscopic renal damage, arterial pressure, and renal hemodynamics in Dahl salt-resistant (R) and S rats over a 5-wk period of high Na intake with and without antioxidant treatment.

METHODS

Animal protocol and experimental measurements. Five-week experiments were performed on four groups of 7- to 8-wk-old Dahl S and Dahl R rats (Rapp strain; Harlan Sprague Dawley, Indianapolis, IN) with the authorization of the Institutional Animal Committee, which ensures that all possible steps are taken to avoid animals’ suffering at each stage of the experiment. Rats were randomly divided into four groups: Dahl S rats with 8% Na (n = 8), Dahl S rats with 8% Na + vitamin C/E (n = 8), Dahl R rats with 8% Na (n = 6), and Dahl R rats with 8% Na + vitamin C/E (n = 6). Vitamin C (1g/l) was administered in the drinking water and vitamin E (5,000 IU/kg food) in the rat chow. After 3 wk on the diets, catheters were implanted into the femoral artery and vein using isoflurane anesthesia (1%). After 4 days of recovery, a 10-day period of collection of arterial pressure, heart rate, and renal hemodynamics data began as has been done before by our group (22, 43, 45, 46). GFR and effective renal plasma flow (ERPF) were determined in conscious rats on day 34, as has been done before by our group (21, 44). Briefly, iothalamate and aminohippurate concentrations of a 4-h-fasted plasma sample were measured after a 12-h period of intravenous infusion of [125I]iothalamate and aminohippurate sodium (4, 8).

An additional 36 rats were subjected to the same diet regimen as the above groups for 5 wk, but without catheters, to provide additional renal tissues for several assays. At the end of week 5, rats were...
anesthetized with isoflurane and kidneys were removed. The renal cortex and medulla were isolated and homogenized in appropriate buffers and protease inhibitors. Before the kidney tissues were removed, blood pressures were measured under isoflurane anesthesia to ensure that their pressures were not different from that of rats in the catheter groups. Chemiluminescence of renal tissues was measured with 5 µM lucigenin (bis-N-methylacridinium nitrate) using a Berthold Autolumat Plus luminometer. The accuracy of measuring oxygen free radical production with 5 µM lucigenin has been validated with electron spin resonance methods (20, 37), and in renal tissue this method produces good signals without addition of reactive oxygen species (ROS) substrates, as previously shown (16, 42). Protein amounts in renal tissues and urine were quantified with the Lowry assay and the Bradford assay, respectively.

**RESULTS**

Rats were fed the high Na diet for 5 wk with 5 control groups. Chemiluminescence of renal tissues was measured to ensure that their pressures were not different from that of rats in the control groups. Renal cortical and medullary GSH/GSSG and renal cortical hydrogen peroxide in Dahl R rats with 8% Na (n = 6), Dahl S rats with 8% Na + vitamins (n = 6), Dahl S rats with 8% Na + vitamins (n = 6), and S rats with 8% Na + vitamins (n = 7) were measured using kits from R&D Systems according to the manufacturer’s instructions. Renal cortical MCP-1 was determined using a kit from Biosource.

**Measurement of renal glutathiones, H2O2, IL-6, TNF-α, and MCP-1.** Reduced glutathione (GSH) and oxidized glutathione (GSSG) were measured with the fluorescent detection of dansyl derivatives using HPLC according to the method of Jones et al. (17), as has been done before by our group (46). An Amplex red kit from Molecular Probes was used to measure H2O2 in the renal cortex in all groups. Renal cortical IL-6 and TNF-α were measured using kits from R & D Systems according to the manufacturer’s instructions. Renal cortical MCP-1 was determined using a kit from Biosource.

**Measurement of renal monocytes/macrophages and NF-κB activation.** Monocytes/macrophages in the kidney sections collected at 5 wk of the various diets were measured with an indirect immunoperoxidase methodology (29) using ED-1, a monoclonal antibody to monocytes/macrophages (Chemicon). NF-κB activation was measured in whole kidney nuclear protein extracts with an electrophoretic mobility shift assay (EMSA) using a modification of a previous method (19). EMSA detection was performed using a Gelshift NF-κB p65 kit (Active Motif, Carlsbad, CA) according to the manufacturer’s procedures.

**Analysis of glomerular and tubulointerstitial injury.** Kidney sections from each rat were examined for necrotic and sclerotic glomeruli at ≥200 magnification using periodic acid-Schiff-hematoxylin stain (21, 47). A Masson trichrome-stained kidney section from each rat was analyzed for tubulointerstitial renal injury. This injury was measured as the proportion of the number of points in interstitial tissue with increased amounts of blue staining, dilated cast-containing tubules, or tubules showing acute injury divided by the number of points on the grid overlying nonglomerular and nonvascular cortex.

**Statistical analysis.** Comparison of data from S and R rats on high Na with and without vitamins were first performed using analysis of variance, followed by a Fisher least significant difference test for post hoc analysis. Differences were considered to be statistically significant if P < 0.05. All data are means ± SE.

**RESULTS**

Renal cortical and medullary GSH/GSSG and renal cortical H2O2 responses to high Na and vitamins. Figure 1 shows that Dahl S rats on high Na intake had a significantly lower GSH/GSSG ratio in the renal cortex and medulla compared with Dahl R rats on the same diet. This ratio has been shown to be a highly reliable index of oxidative stress (17). Treatment of high-Na Dahl S rats with vitamins C and E resulted in a significantly higher GSH/GSSG ratio in the cortex and medulla. Figure 1 also shows that renal cortical H2O2 concentration was higher in high-Na Dahl S rats compared with high-Na Dahl R rats, and vitamin treatment in Dahl S rats caused a significantly lower H2O2 level.

Renal NF-κB and renal cortical O2− release responses to high Na and vitamins. Figure 2A shows a representative EMSA gel blot for Dahl S rats on high Na or high Na + vitamins. Figure 2, A and B, shows average gel blot data for Dahl S and R rats with or without vitamin treatment. Renal NF-κB was significantly lower in high-Na Dahl S rats treated with vitamins C and E, but the vitamins did not significantly change NF-κB in Dahl R rats. EMSA determination of NF-κB was done for all Dahl R groups separately from that for the Dahl S rats; therefore, because of different activities of the 32P isotope used in each run, values from Dahl R rats cannot be directly compared with those from Dahl S rats. Figure 2D indicates that Dahl S rats on high Na intake had significantly higher O2− release in the renal cortex compared with Dahl R rats on the same diet. Vitamin C and E treatment significantly decreased O2− release from renal cortical tissue in high-Na Dahl S rats but not in Dahl R rats.

**Renal monocyte/macrophage responses to high Na and vitamins.** The four images at the top of Fig. 3 are representative renal micrographs of the four groups of rats showing ED-1+ cells (monocytes/macrophages). The graph in Fig. 3 shows the average ED-1+ cells for the glomerulus, cortex, or medulla of each group and indicates that renal cortical monocyte/macrophages reached a value of 8.7 ± 0.5 cells/mm² in high-Na Dahl S rats compared with 2.4 ± 0.2 cells/mm² in high-Na Dahl R rats (P < 0.05). Treatment of high-Na Dahl S rats with...
vitamins resulted in a significantly lower value of 4.3 ± 0.3 cells/mm² in the cortex.

**IL-6, TNF-α, and MCP-1 responses to high Na and vitamins.** Figure 4 shows that the IL-6 levels in the renal cortex of high-Na Dahl S rats were significantly higher than those of high-Na Dahl R rats. However, vitamin treatment in Dahl S rats did not lower the IL-6 levels. Renal cortical TNF-α levels were significantly higher in high-Na Dahl S rats compared with high-Na Dahl R rats, and TNF-α levels were lower after vitamin treatment in Dahl S rats but not in Dahl R rats. Renal cortical MCP-1 levels were higher in Dahl S rats compared with Dahl R rats, and vitamin treatment resulted in significantly lower values in Dahl S rats but not in Dahl R rats.

**Mean arterial pressure, heart rate, and urinary protein excretion responses to high Na and vitamins.** Figure 5 shows that vitamin treatment markedly decreased arterial pressure in the high-Na Dahl S rats. The arterial pressures are from the last 10 days of the 5-wk experiment and reached a value of 191 ± 5 mmHg in the high-Na Dahl S rats compared with 92 ± 1 mmHg in the high-Na Dahl R rats on day 35 (P < 0.05). Heart rate decreased significantly on days 26–29 and 32–34 in the high-Na + vitamin Dahl S rats, compared with the high-Na Dahl S rats. Figure 5 also shows that throughout the last 10 days of the experiment, the high-Na Dahl S rats had elevated values of urinary protein excretion compared with high-Na Dahl R rats. Vitamin treatment decreased urinary protein excretion in the high-Na Dahl S rats, suggesting that antioxidant treatment prevented renal damage in high-Na Dahl S rats.

**GFR and renal plasma flow responses to high Na and vitamins.** Figure 6 shows that a high Na intake in Dahl S rats caused a significantly lower GFR (1.5 ± 0.1 ml/min) compared with either the Dahl R high-Na rats (3.2 ± 0.2 ml/min) or the Dahl S high Na + vitamin rats (2.8 ± 0.2 ml/min). Therefore, vitamin treatment prevented a decrease in GFR in Dahl S rats on high Na intake. Also, compared with R rats, high Na intake in S rats caused a significant decrease in ERPF to 3.6 ± 0.7 ml/min, and vitamin treatment significantly increased ERPF to 6.3 ± 0.7 ml/min. Renal vascular resistance was 30.5 ± 4.8 mmHg·ml⁻¹·min in high-Na Dahl R rats and 104.4 ± 12.6 mmHg·ml⁻¹·min in high-Na Dahl S rats (P < 0.05). Vitamin treatment in Dahl S rats resulted in a resistance value of 47.3 ± 7.2 mmHg·ml⁻¹·min (P < 0.05 compared with Dahl S high-Na rats).
Average glomerular and tubulointerstitial damage responses to high Na and vitamins. As shown in Fig. 7, tubulointerstitial damage averaged 0.35 ± 0.08 (fractional area) in the high-Na Dahl S group, and this area decreased significantly in high-Na Dahl rats on vitamin treatment. Figure 7 also shows that the average glomerular necrosis significantly increased in the high-Na Dahl S rats compared with either the Dahl R high-Na rats or Dahl S high-Na vitamin groups. The antioxidant vitamin treatment decreased the amount of glomerular necrosis in the Dahl S rats on a high Na intake by 83%.

DISCUSSION

Compared with levels in Dahl R rats, increases in dietary Na intake in Dahl S rats caused significant increases in ROS, renal cytokines, chemokines, monocyte/macrophage infiltration and increased blood pressure, renal damage, and dysfunction. For the first time, our data show that in Dahl S rats on high Na intake, the elevated levels of renal oxidative stress, NF-κB, cytokines, chemokines, arterial pressure, and renal infiltration of immune cells were reduced by antioxidant treatment. In particular, new information from this study shows that antioxidant treatment in Dahl S rats reduced renal H$_2$O$_2$ and increased GSG/GSSG, reduced NF-κB and renal monocyte/macrophage infiltration in the glomerulus, cortex, and medulla, and lowered renal TNF-α, MCP-1, and renal damage. These data suggest that increases in renal oxidative stress may contribute to the increases in renal inflammation seen in Dahl salt-sensitive hypertension.

In the present study, oxidative stress increased in the kidney of high-Na Dahl S rats as evidenced by decreases in the GSH/GSSG ratio and increases in renal cortical O$_2$•⁻ release and renal cortical H$_2$O$_2$ production. Vitamin C and E treatment increased the GSH/GSSG ratio in Dahl S rats but did not significantly change this ratio in Dahl R rats. Vitamin treatment also decreased the elevated production of H$_2$O$_2$ and O$_2$•⁻ in Dahl S rats but not in Dahl R rats. Although both Dahl R and S rats were on a high-Na diet, renal oxidative stress was higher in Dahl S rats compared with Dahl R rats, and the vitamin treatment was effective in reducing ROS only in the hypertensive Dahl S rats.

ROS have been shown to induce activation of transcription factors such as NF-κB and activator protein-1 (11). In particular, micromolar concentrations of H$_2$O$_2$ cause an activation of
and NF-κB activation also occurred (30). Sustained use of the antioxidant pyrrolidinedithiocarbamate (PDTC) in SHR prevented the increase in arterial pressure and significantly decreased renal immune cell invasion (28). The antioxidants PDTC and 4-hydroxy-2,2,6,6-tetramethyl piperidinoxyl (Tempol) were given to DOCA-salt hypertensive rats, and decreases occurred in blood pressure, renal monocyte/macrophage infiltration, and NF-κB activation (6). The present study and the above cited studies indicate that increases in ROS can increase renal infiltration of immune cells, resulting in renal damage. Antioxidant treatment effectively reduces renal inflammation, thus decreasing renal immune cells.

A second goal of this study was to determine whether renal inflammation occurs only in hypertensive Dahl rats. Indeed, this did occur, since only the hypertensive S rats had evidence of inflammation. For instance, renal IL-6, TNF-α, MCP-1, and monocyte/macrophage count were elevated in Dahl S rats compared with Dahl R rats. These variables were lower in the antioxidant-treated groups with the exception of IL-6, and renal NF-κB also decreased. We do not have direct evidence that high blood pressure is necessary for renal inflammation to occur in Dahl S rats. However, a previous study in our laboratory (21) showed that increases in high blood pressure indeed preceded renal damage. Therefore, there is a possibility NF-κB (11) by phosphorylating the IκB protein. Würzburg T cells have been shown to produce enhanced amounts of NF-κB when exposed to small amounts of H₂O₂ (2). H₂O₂ activates DNA binding of NF-κB in vivo but not in vitro (35). Also, increased superoxide dismutase levels in JB6 cells, which should increase H₂O₂ levels, potentiated the NF-κB response to TNF-α (34). In the present experiment, renal H₂O₂ levels were elevated in high-Na Dahl S rats and are a likely source of NF-κB activation. Indeed, when vitamins C and E were placed in the diet of these high-Na Dahl S rats, both renal H₂O₂ and NF-κB levels decreased.

NF-κB activation rapidly induces the transcription of proinflammatory cytokines, chemokines, and adhesion molecules (23, 32, 36). In the present study in high-Na Dahl S rats, renal IL-6 and TNF-α increased compared with levels in Dahl R rats. Also, the chemokine MCP-1 increased, and this was associated with increased renal monocyte/macrophage infiltration. These inflammatory changes in TNF-α and MCP-1 were alleviated in Dahl S rats by addition of vitamins C and E to their diet.

Several studies have shown that antioxidants will reduce hypertension-induced inflammatory changes. Antioxidants were given to 7-mo-old SHR, and the hypertension and renal immune cell infiltration decreased (33). Immune cells accumulated at the onset of SHR hypertension after only 3 wk of age,

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creases in inflammatory cytokines and chemokines. The O$_2^•$ release may cause increases in renal vascular resistance and decreases in renal hemodynamics in Dahl S hypertension and is increased in Dahl S hypertension due to higher plasma levels. Vitamin treatment can prevent renal damage in Dahl S hypertension, and this can be increased by vitamin treatment in Dahl S rats. Another mechanism that could reduce renal hemodynamics is the nonenzymatic oxidation of arachidonic acid by ROS, resulting in the formation of vasoconstrictor isoprostanes (22). Intrarenal angiotensin II has been shown to be increased in Dahl S hypertension (18), and this could be caused by angiotensin II formation in immune cells infiltrating into the renal interstitium (32). The angiotensin could cause renal vasoconstriction and renal Na retention. Finally, Dahl salt-sensitive hypertension has been associated with considerable renal damage with nephron destruction (43), which could cause decreases in renal vascular resistance and decreases in GFR and renal plasma flow.

Vitamin treatment in Dahl S rats on a high-Na diet caused significant decreases in renal damage as evidenced by large decreases in urinary protein excretion and pronounced decreases in tubulointerstitial and glomerular damage. In fact, the glomeruli of the high-Na Dahl S rats experience severe glomerular necrosis, as has been shown before by our group (43, 45, 46). Thus the renal changes in the high-Na Dahl S rats have an appearance similar to that in kidneys from patients with florid malignant hypertension. Our group (43) also has found that antioxidant treatment with vitamins C and E of high-Na Dahl S rats significantly attenuated the glomerular and arteriolar necrosis and shifted the acute tubulointerstitial injury away from progressively deteriorating acute injury and toward more stable chronic renal injury. Renal damage remained at low levels in Dahl R rats and was unaffected by vitamin treatment.

Vitamins C and E have been used alone or in combination to prevent renal damage or increases in blood pressure in rats. Results from these studies were used to choose doses of the vitamins in our study with a goal of reducing renal damage and arterial pressure. Arterial pressure was reduced in Sprague-Dawley rats on a high-Na diet with 100 mg/kg of vitamin C (25). In the present study we used a comparable dose of ~100 mg/day of vitamin C. In aging rats, vitamin E at 5,000 IU/kg food was used, and plasma vitamin E increased by 50%, GFR increased, and F$_2$-isoprostanes in renal tissue decreased (26). The same dose of vitamin E in rat chow was used in the present study. The doses of vitamin E used in experimental studies on animals were much higher than those used in typical clinical studies (1, 10, 26). One reason is that the basal dietary requirement vitamin E in rats, as recommended by the American Institute of Nutrition, is seven times higher than in humans (1). In a new study in hypercholesterolemic patients (27), significant decreases in plasma isoprostane were realized only when vitamin E intake was increased 25–100 times over the recommended daily amount. In the present study, vitamin E intake was increased ~100 times over the basal recommended intake in rats.

Several clinical trials have been designed to determine whether vitamin treatment can improve cardiovascular outcomes. Although results have been variable, some studies have...
shown that treatment of hypertensive patients with vitamin C lowers blood pressure (12). Clinical studies on vitamin E typically use doses equal to or less than 400 IU/day, and little reduction in cardiovascular risk has been found (14); however, when 800 IU/day of vitamin E was used (7, 38), significant decreases in cardiovascular risk were noted. Recently, a 16-wk study in hypercholesterolemic patients showed that 400 IU/day of vitamin E did not decrease serum levels of F$_2$-isoprostanes, but doses of 800–3,200 IU/day caused significant reductions (27). A second possible reason why vitamin E showed little cardiovascular benefit in some clinical studies is that vitamin E can become a free radical in the body, but vitamin C converts the pro-oxidant vitamin E radical back to vitamin E (9), which is the reason we used vitamin C in our study. A third reason why vitamin therapy was possibly ineffective in clinical studies is that the patients entered the studies with preexisting conditions. You can refer to the original text for a detailed explanation of these points.

In conclusion, antioxidant treatment with vitamins C and E in high-Na Dahl S rats for 5 wk decreased renal inflammatory cytokines and chemokines, renal immune cells, NF-$kappa$B, and arterial pressure and improved renal function and damage. Our data suggest that oxidative stress in the hypertensive Dahl S rat leads to significant stimulation of renal inflammation, and this is associated with renal damage and dysfunction, which is alleviated with antioxidant treatment. There was little evidence of renal inflammation in the normotensive Dahl R rat and thus little renal damage or dysfunction, suggesting that arterial pressure must be initially elevated to cause renal damage, oxidative stress, and inflammation.

GRANTS
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REFERENCES
30. Rodriguez-Iiturbe B, Vaziri ND, Herrera-Acosta J, Johnson RJ. Oxidative stress, renal infiltration of immune cells, and salt-sensitive hyper-


