Renal NF-κB activation and TNF-α upregulation correlate with salt-sensitive hypertension in Dahl salt-sensitive rats

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Gu, Jian-Wei, Niu Tian, Megan Shparago, Wei Tan, Amelia P. Bailey, and R. Davis Manning Jr. Renal NF-κB activation and TNF-α upregulation correlate with salt-sensitive hypertension in Dahl salt-sensitive rats. Am J Physiol Regul Integr Comp Physiol 291: R1817–R1824, 2006. First published July 13, 2006; doi:10.1152/ajpregu.00153.2006.--- Molecular mechanisms of salt-sensitive (SS) hypertension related to renal inflammation have not been defined. We seek to determine whether a high-salt (HS) diet induces renal activation of NF-κB and upregulation of TNF-α related to the development of hypertension in Dahl SS rats. Six 8-wk-old male Dahl SS rats received a HS diet (4%) and, six Dahl SS rats received a low-sodium diet (LS, 0.3%) for 5 wk. In the end, mean arterial pressure was determined in conscious rats by continuous monitoring through a catheter placed in the carotid artery. Mean arterial pressure was significantly higher in the HS than the LS group (177.9 ± 3.7 vs. 109.4 ± 2.9 mmHg, \( P < 0.001 \)). There was a significant increase in urinary albumin secretion in the HS group compared with the LS group (22.3 ± 2.6 vs. 6.1 ± 0.7 mg/day; \( P < 0.001 \)). Electrophoretic mobility shift assay demonstrated that the binding activity of NF-κB p65 proteins in the kidneys of Dahl SS rats was significantly increased by 53% in the HS group compared with the LS group (\( P = 0.007 \)). ELISA indicated that renal protein levels of TNF-α, but not IL-6, interferon-γ, and CCL28, were significantly higher in the HS than the LS group (2.3 ± 0.8 vs. 0.7 ± 0.2 pg/mg; \( P = 0.036 \)). We demonstrated that plasma levels of TNF-α were significantly increased by fivefold in Dahl SS rats on a HS diet compared with a LS diet. Also, we found that increased physiologically relevant sodium concentration (10 mM) directly stimulated NF-κB activation in cultured human renal proximal tubular epithelial cells. These findings support the hypothesis that activation of NF-κB and upregulation of TNF-α are the important renal mechanisms linking proinflammatory response to SS hypertension.

nuclear factor-κB; tumor necrosis factor-α; kidneys; inflammation

HYPERTENSION HAS BEEN RECOGNIZED as a multifactorial trait resulting from the effects of a combination of both environmental and genetic factors. An excess of dietary salt is the most common environmental factor that contributes to the pathogenesis of hypertension (2, 11, 13, 37). Salt-sensitive (SS) hypertension plays a significant role as a factor contributing to the manifestation and progression of cardiovascular and chronic renal diseases (7, 16–17). About 50% of patients with essential hypertension have a SS hypertension, in which the subgroups of patients exhibit salt sensitivity and more severe progression of hypertensive target-organ damage, including end-stage renal disease (7, 22, 26, 44). However, the mechanisms of SS hypertension and the related renal injury are not well established.

Recent evidence suggests that renal proinflammatory response, such as immune cell accumulation in the kidneys, may play an important role in mediating sodium retention and, thereby, in the development of hypertension (33). The genetic model of the Dahl SS rat displays SS hypertension and related target-organ damage and has been widely used for investigating the human polymorphisms of SS hypertension (8, 15, 23–24, 32). In the Dahl model of hypertension, a high-salt (HS) diet significantly increases total circulating leukocyte counts in the Dahl SS rats but not in the Dahl salt-resistant rats (38). A recent report has indicated that a HS diet increases tubulointerstitial infiltration of inflammatory cells in Dahl SS rats (39). Several studies have also shown that reducing renal inflammatory cell infiltrate prevents the development of SS hypertension (1, 34). Despite the evidence linking proinflammatory response to SS hypertension, the molecular mechanisms of this relationship have yet to be defined.

Many cellular genes involved in the early process of immunity, acute phase, and inflammatory responses are regulated at the level of transcription by the transcription factor nuclear factor-κB (NF-κB) (3, 5). NF-κB activates numerous proinflammatory genes, including tumor necrosis factor-α (TNF-α), interleukin (IL)-6, interferon-γ, and CC chemokines such as CCL28 (4, 12, 28, 43). Based on all findings mentioned above, we hypothesize that renal proinflammatory response characterized by the alteration of multiple inflammatory factors may play an important role in the pathogenesis of SS hypertension. In the present study, we seek to determine whether a HS diet induces renal activation of NF-κB and upregulation of multiple proinflammatory factors, including TNF-α and interferon-γ, in relation to the development of hypertension and renal injury in Dahl SS rats.

MATERIALS AND METHODS

Animal protocol and experimental measurements. The protocols were carried out according to the guidelines for the care and use of laboratory animals implemented by the National Institutes of Health and the Guidelines of the Animal Welfare Act and were approved by the University of Mississippi Medical Center’s Institutional Animal Care and Use Committee. Experiments were performed in 12 conscious 7- to 8-wk-old male Dahl SS rats, Rapp strain (Harlan Sprague Dawley, Indianapolis, IN). Rats were received at 6–7 wk of age and were allowed to acclimate for 1 wk with standard rat diet (Teklad, Harlan Sprague Dawley) and tap water before beginning the experiment. Rats were placed in a temperature-controlled room with a 12:12-h light-dark cycle. During the 5-wk experimental period, the LS group (\( n = 6 \)) received a LS (0.3%) diet (Teklad, Harlan Sprague Dawley), and the HS group (\( n = 6 \)) received a HS (4%) diet (Teklad, Harlan Sprague Dawley). After 4 wk on the various diets, rats were placed in the metabolic cages, and water intake and urinary volume output were measured daily. Twenty-four-hour urine samples were
collected daily, and urine sodium concentration was determined by flame photometry. Urine protein concentration was determined using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA). Urine albumin concentration was measured using a Neprhat ELISA Kit (Exocell, Philadelphia, PA). At the end of the 5-wk period, rats were anesthetized by isoflurane inhalation using a gas vaporizer (Harvard Apparatus, Holliston, MA). With the use of aseptic techniques, a micro-catheter was placed into the carotid artery and routed under the skin to the back of the neck and secured. Two days after the rats recovered from the surgery, the catheter was connected to a computerized monitoring system, called PowerLab (AD Instruments, Castle Hill, Australia), to continuously record mean arterial pressure (MAP) and heart rate (HR) in the conscious rats 4 h/day for 2 days. The measurements of blood pressure (BP) and HR in each rat were made simultaneously, and all of the values for each rat represented the average of the 4 h/day for 2 days measurements. At the end of experiment, blood samples were collected through the catheters, and again the rats were anesthetized with isoflurane, and the kidneys were removed for various analyses.

Electrophoretic mobility shift assay. Tissue nuclear protein extracts and electrophoretic mobility shift assay (EMSA) for NF-κB were performed by a previous method (20) with some modification. Frozen whole kidneys were pulverized in liquid nitrogen with mortar and pestle. Tissue (200 mg) from each kidney was resuspended in 1.5 ml hypotonic buffer containing protease inhibitor cocktail composed of 10 mmol/l HEPES-KOH, pH 7.9, 10 mmol/l KCl, 1.5 mmol/l MgCl2, and 0.5 mmol/l DTTO, followed by incubation for 15 min on ice. The tissue was homogenized with 10 strokes in the presence of 1% Nonidet P-40. The cytoplasmic fraction and nuclear fraction were collected using a nuclear extract kit (Active Motif, Carlsbad, CA), according to the manufacturer’s procedures. The supernatant was aliquoted and frozen at −80°C until use. The protein concentration for EMSA was quantified by the Bradford method. EMSA detection was performed using a Gelshift NF-κB p65 Kit (Active Motif), according to the manufacturer’s procedures. Nuclear extract (5 μg) was incubated in binding reaction mixture with [32P]dATP end-labeled oligonucleotide containing the NF-κB p65 binding site at 4°C for 30 min. The competition experiments were conducted by adding excess unlabeled wild-type or mutant NF-κB oligonucleotide (Active Motif). The DNA-protein complexes were electrophoresed on a 5% polyacrylamide gel 0.5× Tris-boric acid buffer, dried, and autoradiographed. The intensity of the bands was analyzed with a PhosphorImager (Molecular Dynamics).

NF-κB motif binding assay. We used 10 μg of the nuclear extract from each kidney in the TransAM NF-κB p65 kit (Active Motif), which can measure the binding of activated NF-κB to its consensus sequence attached to a microwell plate, according to the manufacturer’s instructions. We tried to examine whether similar results could be observed using two different methods.

Measurement of protein levels of various cytokines by ELISA. Protein levels of TNF-α, IL-6, interferon-γ, and CCL28 in the plasma and whole kidney tissue of Dahl SS rats were determined using various ELISA kits (R&D Systems, Minneapolis, MN), according to the manufacturer’s instructions. Tissue samples of whole kidney were homogenized in ice-cold PBS buffer containing protease inhibitor cocktail (50 mg wet wt/ml), and the total proteins were extracted using NE-PER Cytoplasmic Extraction Reagents (Pierce, Rockford, IL), according to the manufacturer’s protocol. Using these extraction reagents did not affect the measurements of tissue proteins of those cytokines compared with other regular protein extraction methods. These reagents had no significant effect on the standard curve of those cytokines either. The total protein concentration of tissue supernatant was determined using a Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). The protein levels of those cytokines in the tissues were normalized and expressed as picograms per milligram total tissue protein.

Histological analysis by light microscopy. Midcoronal sections of the left kidneys collected from both LS-fed and HS-fed groups were fixed in buffered formalin and embedded in paraffin for histological studies, as previously described (24, 39). Samples were cut into 3-μm sections and stained with periodic acid-Schiff reagent (PAS) followed by hematoxylin counterstaining. All sections were analyzed by semi-quantitative histological grading (0 = absent, 1+ = mild, 2+ = moderate, and 3+ = severe) for the severity of tubulointerstitial infiltration with inflammatory cells, focal segmental glomerulosclerosis, tubulointerstitial injury, and extracellular matrix expansion.

Exposure to HS in cultured human renal proximal tubular epithelial cells. The human renal proximal tubular epithelial cells (HRPTEC) (HK2) were obtained from the American Type Culture Collection (Rockville, MD). HK2 cells were seeded into T-75 flasks using M199 media (GIBCO) supplemented with 10% FBS (HyClone), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B, and incubated at 37°C in a humidified 5% CO2/air-injected atmosphere. The different sodium concentrations in the media were adjusted by a fixed sodium bicarbonate content and additional sodium chloride (13). The sodium concentrations were 140 mmol/l in the standard media and 150 mmol/l in the HS media, respectively. When the monolayers of cultured HRPTEC reached ~80% confluence, the standard media were replaced by the HS media for an additional 48 h of incubation. The control group was still cultured in the standard media. Then the cells were harvested, and the nuclear proteins were extracted using a nuclear extraction kit (Active Motif), according to the manufacturer’s procedures for determining NF-κB activation using EMSA.

Statistical analyses. All determinations were performed in duplicated sets. Where indicated, data are presented as means ± SE. Statistically significant differences in mean values were tested by an unpaired Student’s t-test. A value of P < 0.05 was considered statistically significant. All statistical calculations were performed using SPSS software (SPSS, Chicago, IL).

RESULTS

Increased MAP, urine protein, and albumin secretion in response to a HS diet. Figure 1 demonstrates that 5 wk of HS diet significantly increases MAP in conscious Dahl SS rats in which it is associated with an increase in total urinary protein secretion as well as albuminuria, compared with the LS rats. MAP increased more than 68 mmHg in the HS group, compared with the LS group (177.9 ± 3.7 vs. 109.4 ± 2.9 mmHg, P < 0.001) (Fig. 1A). There was no significant difference in the HRs between HS and LS groups (582 ± 32 vs. 537 ± 61 beats/min; P = 0.614). The urinary protein secretion, an index of renal damage, increased by fivefold in the HS group, compared with the LS group (168.1 ± 20.2 vs. 33.3 ± 9.2 mg/day; P < 0.01) (Fig. 1B). Consistent with the total urinary protein, there was a significant increase in albuminuria in the HS group, compared with the LS group (22.3 ± 2.6 vs. 6.1 ± 0.7 mg/day; P < 0.001) (Fig. 1C).

Renal activation of NF-κB due to a HS diet. We further investigated the association between hypertension and renal activation of NF-κB in Dahl SS rats. Using EMSA, we found a 53% increase in renal NF-κB p65 binding activity of Dahl SS rats on 5 wk of HS diet, compared with LS diet (P < 0.05) (Fig. 2A and B). Figure 2C illustrates the competition experiments in which the signal of renal NF-κB p65 binding activity is completely blocked by adding excess, unlabeled, wild-type but not mutant NF-κB p65 oligonucleotide (Active Motif). The competition experiments confirm the specific binding activity of renal NF-κB p65. Consistent with EMSA data, TransAM
NF-κB p65-binding assay (Active Motif) indicates that there is a 45% increase in binding of activated renal NF-κB p65 to its consensus sequence in the HS group, compared with the LS group (P < 0.05) (Fig. 2D). In addition, we found that there was no significant difference in renal NF-κB activation between 10-wk-old and 15-wk-old Dahl SS rats with a 0.3% sodium diet.

Effects of a HS diet on renal protein levels of various proinflammatory cytokines. Using ELISA kits, we examined renal protein levels of several NF-κB-related proinflammatory cytokines including TNF-α, IL-6, interferon-γ, and CCL28, in Dahl SS rats on a HS vs. a LS diet. Figure 3A shows that renal protein levels of TNF-α are significantly increased by more than threefold in Dahl SS rats on a HS diet, compared with a LS diet (2.3 ± 0.8 vs. 0.7 ± 0.2 pg/mg; P = 0.036). A HS diet increased renal protein levels of interferon-γ in Dahl SS rats from the basal levels of 1.3 ± 0.5 pg/mg (LS diet) to 3.9 ± 1.9 pg/mg (HS diet), but the difference was not statistically significant (P = 0.15). There were no significant changes of renal protein levels of IL-6 or CCL28 between the HS and LS groups: 8.2 ± 1.6 vs. 11.6 ± 2.4 pg/mg (P = 0.09) and 28.4 ± 7.2 vs. 28.7 ± 6.9 pg/mg (P = 0.97), respectively.

Effects of a HS diet on plasma levels of various proinflammatory cytokines. Figure 3B indicates that plasma levels of TNF-α are increased by more than fivefold in Dahl SS rats on a HS diet, compared with a LS diet (5.4 ± 0.5 vs. 0.9 ± 0.2 pg/ml; P < 0.01). However, there were no significant changes in plasma levels of IL-6, interferon-γ, and CCL28 in Dahl SS rats on HS and LS diets, and their values were 60.8 ± 27.5 vs. 45.8 ± 17.1 pg/ml (P = 0.67), 6.8 ± 0.7 vs. 11.6 ± 6.5 pg/ml (P = 0.67), and 3.4 ± 0.6 vs. 3.9 ± 1.2 pg/ml (P = 0.67), respectively.

Renal histological changes due to a HS diet. Histological analysis by light microscopy of PAS-stained sections of the kidneys showed that there were no marked renal abnormalities in Dahl SS rats with a LS diet (Fig. 4, A and B). In contrast, Dahl SS rats with a HS diet developed severe glomerulosclerosis and tubulointerstitial injury, and moderate tubulointerstitial infiltration with inflammatory cells, as well as extracellular matrix expansion. The PAS stain findings are consistent with our laboratory’s previous results that Masson’s trichrome stain indicated severe glomerulosclerosis and tubulointerstitial injury in Dahl SS rats on a HS diet (22).

Effect of increased sodium concentration on NF-κB activation in cultured HRPTEC. Using cell cultures, we examined whether increased physiologically relevant sodium concentrations can have a direct cellular effect on NF-κB activation in cultured HRPTEC, which is independent of BP. As shown in Fig. 5, EMSA demonstrated that a physiologically relevant HS concentration (150 mmol/l) directly increased NF-κB binding activity by 46% in cultured HRPTEC, compared with those in normal sodium (140 mmol/l) media (n = 6; P < 0.01; signal intensity 3,247 ± 311 vs. 2,217 ± 214).

DISCUSSION

NF-κB is a key transcription factor in the activation of genes related to proinflammatory response. Several recent studies (1, 33–34, 38–39) have provided convincing evidence that renal proinflammatory response plays an important role in mediating SS hypertension. The major new finding in this study is that 5 wk of HS diet causes renal activation of NF-κB in relation to the development of hypertension and albuminuria in Dahl SS rats. This new evidence supports the hypothesis that activation of NF-κB is one of the important renal mechanisms linking proinflammatory response to SS hypertension.

A question that must be asked is what are the possible mechanisms of renal activation of NF-κB in SS hypertension? Free radicals not only have been shown to be a cause of cell damage but are also involved in a variety of mechanisms that ensure cellular physiological equilibrium, such as regulation of vascular tone, sensing of oxygen tension, and signal transduction. Oxidative stress has been documented in both experimental and human hypertension (36, 45). There is a strong, positive correlation between renal superoxide-positive cells and renal infiltration of macrophages in the spontaneously hypertensive rats (SHR) treated or untreated with mycophenolate mofetil.
Reactive oxygen species accumulation has been reported in deoxycorticosterone acetate-salt hypertension. The production of superoxide radicals in the microvessels of the mesentery and plasma H$_2$O$_2$ concentration were increased in hypertensive Dahl SS rats compared with Dahl salt-resistant rats. Our laboratory has recently reported that oxidative stress characterized by increased renal cortical and medullary O$_2^•$ release contributes to Dahl SS hypertension and the accompanying renal damage. Interestingly, Beswick et al. reported that treatment with pyrrolidinedithiocarbamate, a potent antioxidant, attenuated systolic BP, suppressed renal NF-κB binding activity, and alleviated renal immune cell infiltration in deoxycorticosterone acetate-salt hypertension. Therefore, it is conceivable that oxidative stress may cause renal activation of NF-κB in various hypertensive animals, including Dahl SS hypertensive rats. Further studies are necessary to confirm whether antioxidant treatment attenuates renal activation of NF-κB in relation to reducing BP, renal oxidative stress, and immune cell infiltration in Dahl SS hypertensive rats.

Fig. 2. A and B: electrophoretic mobility shift assay for detection of nuclear factor-κB (NF-κB) shows a 53% increase in renal NF-κB p65 binding activity of Dahl salt-sensitive rats on 5 wk of a high-salt diet, compared with a low-salt diet ($n=6$; *$P<0.05$). C: the competition experiments in which the signal of renal NF-κB p65 binding activity is completely blocked by adding excess unlabeled wild-type but not mutant NF-κB p65 oligonucleotide. D: by using TransAM NF-κB p65-binding assay, there is a 45% increase in binding of activated renal NF-κB p65 to its consensus sequence in the high-salt group, compared with the low-salt group ($n=6$; *$P<0.05$).
The cause-and-effect relationships between HS diet, inflammation, renal injury, and hypertension are very complicated and should be further studied in many different ways. It is very important to provide experimental evidence linking to the cause-and-effect relationships between HS diet, inflammation, renal injury, and hypertension. The related experiments may include investigating the direct effect of sodium intake, the effects of antioxidant treatment, and blocking NF-κB signaling pathway on renal inflammation. More recent evidence suggests that adverse effects of HS intake on renal-cardiovascular systems are independent of BP (11, 13). Therefore, we did an additional experiment to determine whether increased physiologically relevant sodium concentrations directly stimulate NF-κB activation in cultured HRPTEC. We have found that increased physiologically relevant sodium concentrations can have a direct cellular effect on NF-κB activation in cultured HRPTEC, which is independent of BP. This is unique experimental evidence linking to the cause-and-effect relationships between HS diet, inflammation, renal injury, and hypertension.

Also, several studies demonstrated that melatonin reduced renal NF-κB activation, oxidative stress, immune cell infiltration, and hypertension in SHR (29) and that pyrrolinedithiocarbamate (27) or lipoic acid (25) attenuated hypertension, renal oxidative stress, and NF-κB activation in double-transgenic rats harboring human renin and angiotensinogen genes. Thus the phenomena of renal NF-κB activation and inflammatory damage correlate with hypertension in various hypertensive animal models. However, SS hypertension has more severe progression of renal damage even in the early stage of hypertension. In SHR, severe renal damage occurred in the late stage of hypertension. In addition, the mechanisms of renal inflammatory damage may differ between SHR and Dahl SS...
Therefore, our data suggest that HS diet-induced renal activation of NF-κB and upregulation of TNF-α may represent a vicious cycle in which NF-κB and TNF-α promote each other in mediating a progressive renal proinflammatory response in SS hypertension. Future studies are needed to elucidate the effects of NF-κB and/or TNF-α inhibition on breaking this vicious cycle of proinflammatory response in Dahl SS hypertensive rats.

In addition, the present study indicates that plasma levels of TNF-α are increased by more than fivefold in Dahl SS rats on a HS diet, compared with a LS diet. This finding is consistent with a previous report that a HS diet significantly increases total circulating leukocyte counts in Dahl SS rats but not Dahl salt-resistant rats (38). It will be interesting to examine whether circulating TNF-α can be used as a biochemical marker for monitoring inflammatory response in SS hypertension.

NF-κB activates numerous proinflammatory genes, including TNF-α, IL-6, interferon-γ, and CC chemokines such as CCL28 (4, 12, 28, 43). However, in the present study, we found a significant increase in renal TNF-α, an insignificant increase in renal interferon-γ, and no change in renal protein levels of IL-6 or CCL28 in Dahl SS rats following 5 wk of HS diet, compared with LS diet. This discrepancy may be due to the fact that the upregulation of these proinflammatory cytokines may occur at different stages of inflammation. The present study is limited by this single-point measurement. Further studies are needed to examine temporal progression of renal proinflammatory response and hypertension in relation to the activation of various proinflammatory cytokines in Dahl SS rats.

In conclusion, our results indicate that 5 wk of a HS diet causes the activation of NF-κB and the upregulation of TNF-α in the kidneys in association with the development of hypertension, albuminuria, and marked renal histological abnormalities, including tubulointerstitial infiltration with inflammatory cells in Dahl SS rats. We demonstrate that plasma levels of TNF-α are significantly increased in Dahl SS rats on a HS diet, compared with a LS diet. Also, we find that increased physiologically relevant sodium concentration (10 mmol/l) directly stimulates NF-κB activation in cultured HRPTEC. These find-

![Graph showing NF-κB binding activity](http://ajpregu.physiology.org/)

**Fig. 5.** Human renal proximal tubular epithelial cells (HK2) were cultured in the media, having sodium concentrations of 140 and 150 mmol/l for 48 h, respectively. Electrophoretic mobility shift assay demonstrated that a physiologically relevant high-sodium concentration (150 mmol/l) directly increased NF-κB binding activity by 46% in cultured HK2 cells, compared with those in normal sodium (140 mmol/l) media (n=6; *P<0.01; signal intensity 3,247 ± 311 vs. 2,217 ± 214).
ings support the hypothesis that the activation of NF-κB and upregulation of TNF-α are the important renal mechanisms linking proinflammatory response to SS hypertension. Therefore, further studies are necessary to identify the therapeutic targets of NF-κB inhibition and/or TNF-α inhibition in SS hypertension.

GRANTS

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REFERENCES


