Original Article

Albuminuria, Expression of Nicotinamide Adenine Dinucleotide Phosphate Oxidase and Monocyte Chemoattractant Protein-1 in the Renal Tubules of Hypertensive Dahl Salt-Sensitive Rats

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In chronic renal diseases, experimental and human data suggest that excess albumin filtered through the glomerular capillary barrier is over-reabsorbed by proximal tubular cells, thereby activating these cells and upregulating the expression of chemokines. On the other hand, a high-salt diet has been shown to induce proteinuria in hypertensive Dahl salt-sensitive (DSS) rats, accompanied with the expression of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in the kidney. In the current study, we therefore examined albuminuria and the expressions of NADPH oxidase and monocyte chemoattractant protein-1 (MCP-1) in the renal tubular cells in hypertensive DSS rats, as well as the effects of the antioxidant *N*-acetylcysteine (NAC) on each of these parameters. DSS rats were fed a normal-salt diet (0.24% NaCl), a high-salt diet (8% NaCl), or a high-salt diet plus NAC supplementation (15 mg/mL drinking water) for 4 weeks. The high-salt diet provoked an increase in glomerular injuries accompanied with albuminuria and in urinary H_2O_2 and MCP-1 excretion. Immunohistochemical analysis showed the prominent expression of MCP-1 in the dilated tubular cells, where the NADPH oxidase subunit p47phox was also expressed. The current results suggest that albuminuria caused expression of NADPH oxidase and MCP-1 in the dilated renal tubules, resulting in interstitial inflammation and migration of mononuclear cells in DSS rats, because blockade of albuminuria by NAC counteracted the p47phox and MCP-1 expression. (*Hypertens Res* 2007; 30: 991–998)

***Key Words*: Dahl rats, albuminuria, nicotinamide adenine dinucleotide phosphate oxidase, monocyte chemoattractant protein-1, hydralazine**

Introduction

Much evidence suggests that proteinuria must be a mediator of disease progression in chronic glomerular diseases, although proteinuria was originally thought to be merely a marker of the severity of glomerular injury (*1*). Microalbuminuria has been shown to predict future cardiovascular mortality and morbidity in patients with diabetes or hypertension, as well as in subjects in the general population (*2–4*). Experimental data suggest that excess protein such as albumin filtered through the glomerular capillary barrier is over-reabsorbed by human proximal tubular cells, thereby activating them and upregulating the expression of chemokines (*5–7*). Chronic renal diseases that are characterized by glomerular permeability to proteins are accompanied by tubulointerstitial inflammation and migration of mononuclear cells (*1*, *7*). Several *in vitro* studies have demonstrated that albumin

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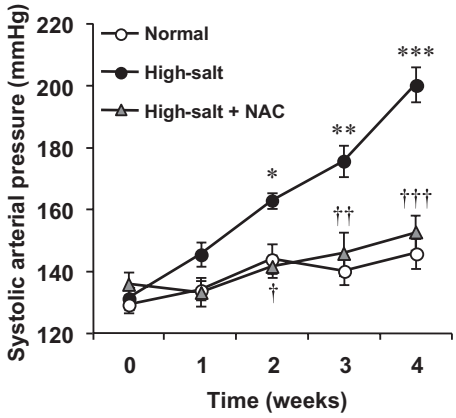

Fig. 1. *Systolic blood pressure (SBP) in Dahl salt-sensitive (DSS) rats. Systolic arterial pressure was measured by the tail cuff method in DSS rats, which received the following diet protocols: normal, 0.24% NaCl diet (n = 6); high-salt, 8% NaCl diet (n = 6); high-salt+NAC, 8% NaCl diet plus N-acetylcysteine supplementation (1.5% in drinking water) (n = 3, except n = 5 at 4 weeks). *p < 0.05, **p < 0.005, ***p < 0.0005 vs. the normal group. †p < 0.05, ††p < 0.005, †††p < 0.0005 vs. the high-salt group.*

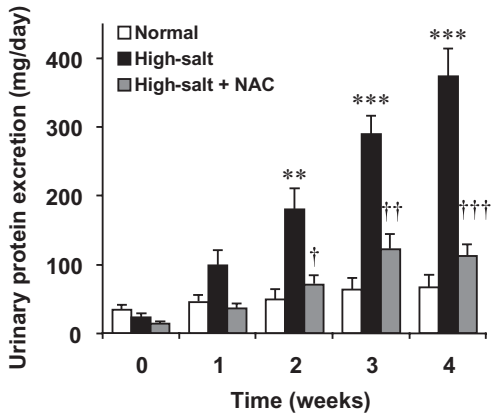

Fig. 2. *Time course of urinary protein excretion in Dahl salt-sensitive rats. Urinary protein concentration was measured by the pyrogallol red method. Normal and high-salt group, n = 6. High-salt+NAC group, n = 3, except n = 8 at 4 weeks. **p < 0.005, ***p < 0.0005 vs. the normal group. †p < 0.05, ††p < 0.005, †††p < 0.0005 vs. high-salt group.*

can stimulate proximal tubular cells to synthesize monocyte chemoattractant protein-1 (MCP-1) (*5*, *6*).

Because a high-salt diet also increases urinary protein excretion in hypertensive Dahl salt-sensitive (DSS) rats (*8*–*10*), we assumed that such a diet would induce MCP-1 expression in the renal tubules of DSS rats. In the current study, renal MCP-1 expression was increased by a high-salt diet in DSS rats, and this effect was counteracted by *N*-acetylcysteine (NAC) supplementation. As expected, NAC supplementation completely blocked the glomerulosclerosis and urinary albumin excretion in the DSS rats in the current study. Expression of MCP-1 is reported to be associated with the redox-sensitive signaling pathway (*11*–*14*). In the present study, we found expression of both nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and MCP-1 protein in the dilated renal tubular cells by immunohistochemistry.

Methods

Animals

Seven-week-old male DSS rats (Dahl S/Jr; KUDO Co., Ltd., Kumamoto, Japan) were fed a normal diet (0.24% NaCl), a high-salt diet (8% NaCl), a high-salt diet plus NAC supplementation (15 mg/mL drinking water), or a high-salt diet plus hydralazine treatment (86 mg/L drinking water) for 4 weeks. Systolic arterial pressure was measured in conscious rats by the tail-cuff method. The rats were anesthetized with intraperitoneal injection of pentobarbital sodium (50 mg/kg body weight) and sacrificed by exsanguination. Tissues were immediately frozen in liquid nitrogen and stored at −80°C. All animal procedures were performed in accordance with the institutional guidelines of Kagawa University.

Measurement of Urinary MCP-1, H_2O_2, Protein and Albumin

Urinary MCP-1 levels were measured by using an enzyme-linked immunosorbent assay (ELISA) kit for rat MCP-1 (BioSource International, Camarillo, USA). Urinary H_2O_2 levels were measured as the amount of peroxidative conversion of nonfluorescent 2′,7′-dichlorodihydrofluorescein diacetate into fluorescent 2′,7′-dichlorofluorescein, where H_2O_2 levels in all samples were expressed as a catalase-inhibitable fraction in the manner described previously (*10*). Urinary protein concentration was measured by the pyrogallol red method (microTP-test; Wako Pure Chemical Industries, Osaka, Japan). Urinary albumin concentration was measured by using a commercially available ELISA kit specific for rat urinary albumin (Nephrat; Exocell Inc., Philadelphia, USA).

Measurement of Superoxide-Producing Activity in the Renal Cortex

NADPH activity in the membrane fraction was measured with an acetylated cytochrome c reduction assay (*10*). The membrane fraction (50 μg protein/mL) was preincubated for 5 min at 37°C with or without 200 units/mL SOD in 0.13 mol/L sodium phosphate buffer, pH 7.0, containing 20 μmol/L acetylated cytochrome c and 0.1 mmol/L diethylenetriamine-pentaacetic acid. NADPH (100 μmol/L) was added at $t = 0$ and absorbance at 550 nm (A_{550}) was serially recorded.

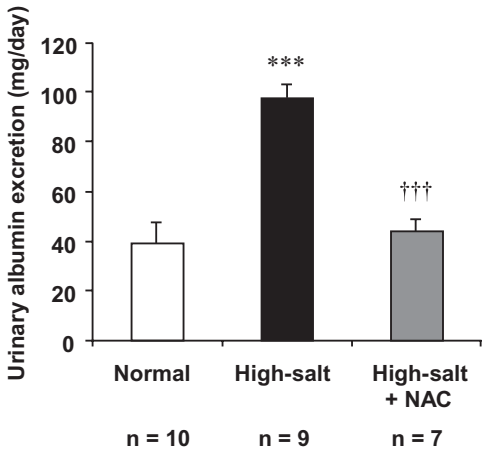

Fig. 3. *Urinary albumin excretion in hypertensive Dahl salt-sensitive rats. The amount of urinary albumin excreted 4 weeks after a high-salt diet was measured by an enzyme-linked immunosorbent assay specific for rat urinary albumin. Values are the mean±SEM. ***$p < 0.0001$ vs. the normal group. †††$p < 0.0001$ vs. the high-salt group.*

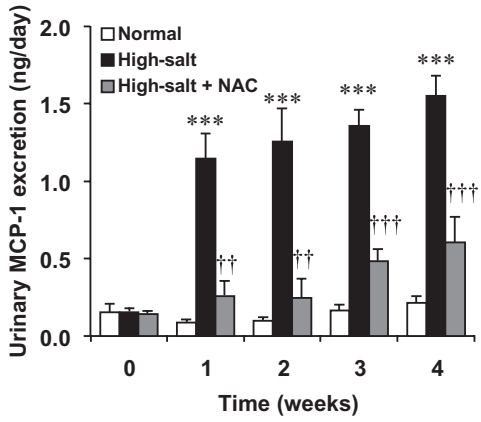

Fig. 4. *Urinary MCP-1 excretion. Urinary concentration of MCP-1 was measured by an enzyme-linked immunosorbent assay. Normal and high-salt group, $n = 6$. High-salt+NAC group, $n = 3$ except $n = 8$ at 4 weeks. ***$p < 0.0005$ vs. the normal group. ††$p < 0.005$, †††$p < 0.0005$ vs. the high-salt group.*

Superoxide-producing activity was determined as the SOD-inhibitable increase in A_{550} in each sample.

Reverse Transcription–Polymerase Chain Reaction Analysis

Total RNA was extracted from the rat renal cortex by the guanidine isothiocyanate method with TRIzol reagent as described in the manufacturer's instructions (Invitrogen, Carlsbad, USA). Reverse transcription–polymerase chain reaction (RT-PCR) analysis was performed as described previously (*10*). Nucleotide sequences of the oligonucleotide primer sets were as follows: for MCP-1, 5′-ATGCAG GTCTCTGTCACG-3′ (forward) and 5′-CTAGTTCTCTGT CATACT-3′ (reverse) (*15*); for G3PDH, 5′-AAACCCATC ACCATCTTCCA-3′ (forward) and 5′-CAGGGGTTTCTT ACTCCTTG-3′ (reverse) (*10*). We optimized the assay conditions and verified that increasing amounts of initial RNA sample yielded increasing amounts of RT-PCR products under these conditions in each primer pair.

Immunohistochemistry

The renal tissue, fixed overnight in 4% paraformaldehyde in 0.1 mol/L phosphate buffer, was embedded in optimal cutting temperature (O.C.T.) compound and quick-frozen in liquid nitrogen. Five μm frozen sections were treated with 3% hydrogen peroxide in methanol for 10 min to inactivate endogenous peroxidases, and incubated with anti-p47phox antibody (1:100 dilution; BD Biosciences, San Jose, USA) (*9*), anti–MCP-1 antibody (1:100 dilution; R-17, Santa Cruz Biotechnology Inc., Santa Cruz, USA) (*16*), or ED1, a monoclonal anti-rat CD68 (marker for tissue macrophages and monocytes) antibody (1:100 dilution; Serotec Ltd., Oxford, UK). After incubation with peroxidase-conjugated secondary antibody (Simple Stain Rat MAX PO; Nichirei Biosciences Inc., Tokyo, Japan), sections were stained with 3′,3′-diaminobenzidine. Mayer's hematoxylin solution was used for counterstaining. As a negative control, sections were stained with antigen-neutralized antibody (for MCP-1) or without primary antibody (for p47phox and CD68).

Histological Analysis

Five μm sections of paraformaldehyde-fixed renal tissues were stained with periodic acid Schiff reagent (PAS) and couterstained with hematoxylin. Tubulointerstitial injury was semiquantitatively graded according to the extension (%) of the damaged areas in the tubules (tubular dilation, tubular atrophy, cast formation, thickening of the tubular basement membrane) and in the interstitium (cellular infiltration, widening) as described by Pichler *et al*. (*17*): 0: no injury; 1+: <10%; 2+: 10–25%; 3+: 26–50%; 4+: 51–75%; 5+: 76–100%. The total cortical area (at least 12 microscopic fields) in each specimen was analyzed and the values of each field were averaged. Glomerulosclerosis was defined by an increase in PAS-positive materials in glomerular tufts and the severity of the lesion was graded according to the percentage of glomerular involvement as described by Raij *et al*. (*18*): 0: no sclerosis; 1+: 1–25% glomerular involvement; 2+: 26–50% glomerular involvement; 3+: 51–75% glomerular involvement; 4+: 76–100% glomerular involvement. In each specimen, the grade of the severity of the lesion (0 to 4) for each glomerulus was totaled and divided by the number of

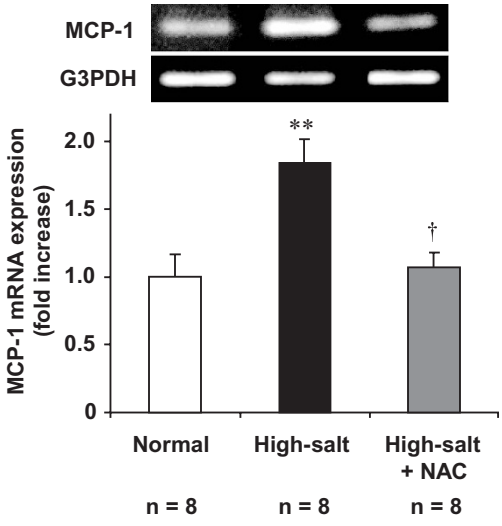

Fig. 5. *Expression of MCP-1 mRNA in the renal cortex. DSS rats received the following protocols for 4 weeks: normal, 0.24% NaCl diet; high-salt, 8% NaCl diet; high-salt+NAC, 8% NaCl diet plus N-acetylcysteine supplementation (1.5% in drinking water). The expression of mRNA was analyzed by RT-PCR and the data is expressed as the fold increase over the normal group. **$p<0.005$ vs. the normal group. †$p<0.05$ vs. the high-salt group.*

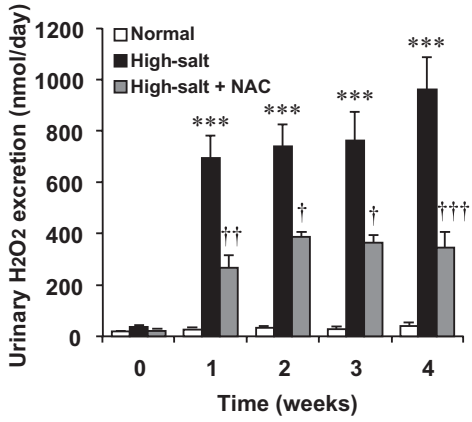

Fig. 6. *Time course of urinary H_2O_2 excretion. Effect of NAC supplementation on urinary H_2O_2 excretion in DSS rats. Urinary concentration of H_2O_2 was determined based on the increase in fluorescence of dichlorofluorescein as described in the Methods section. Normal and high-salt group, $n=6$. High-salt+NAC group, $n=3$ except $n=8$ at 4 weeks. Each data point represents the mean±SEM. ***$p<0.0005$ vs. the normal group. †$p<0.05$, ††$p<0.005$, †††$p<0.0005$ vs. the high-salt group.*

glomeruli examined. The resulting value multiplied by 100 was taken as the glomerulosclerosis score, which ranged from 0 to 400. At least 60 glomeruli in each specimen were analyzed.

Statistical Analysis

Results were presented as the mean±SEM. Data were analyzed with ANOVA followed by the Scheffe or Tukey-Kramer post hoc test. Values of $p<0.05$ were considered to indicate statistical significance.

Results

Systolic arterial pressure was increased progressively over the 4-week experimental period by administration of a high-salt diet in the DSS rats, and this effect was counteracted by NAC supplementation (Fig. 1). Urinary protein excretion per day in the high-salt group was also increased progressively over the 4 weeks (Fig. 2). To determine whether this elevation of urinary protein excretion arose due to the renal glomerular injury, urinary albumin excretion was examined at the 4th week. Urinary albumin excretion per day was increased in high-salt fed rats (Fig. 3). Because cultured renal proximal tubular epithelial cells are reported to secrete MCP-1 in response to albumin (*5*), we speculated that urinary albumin excretion accompanies MCP-1 production in the kidneys of DSS rats.

Urinary MCP-1 excretion was increased 13-fold by a high-salt diet in the first week compared with the normal group (Fig. 4). The levels of MCP-1 excretion in the first week were as high as those in the fourth week. Expression of MCP-1 mRNA in the renal cortex was also increased by the high-salt diet (Fig. 5). NAC supplementation entirely blocked the urinary excretion of MCP-1 and the expression of MCP-1 mRNA, which was consistent with the complete blocking of urinary albumin (Fig. 3) and protein (Fig. 2) excretion by NAC. Exposure to excess proteins in cultured human proximal tubular cells is reported to induce the formation of H_2O_2, which in turn is responsible for the induction of MCP-1 (*6*). Urinary H_2O_2 excretion per day in the high-salt group was increased 19-fold in the first week compared with that in the normal-diet group, and the levels were as high as those in the fourth week (Fig. 6), which was the same as the time-course pattern observed for MCP-1 (Fig. 4). NAC supplementation significantly decreased the urinary excretion of H_2O_2.

Because, in a previous study, Western blot analyses revealed that a high-salt diet induced upregulation of the NADPH oxidase subunits gp91phox and p47phox in the renal cortex of DSS rats, with the latter protein being increased as much as 14-fold (*10*), we here performed immunohistochemical analyses on the expression of the NADPH oxidase subunit p47phox in the renal cortex. Compared with a normal diet (Fig. 7A), the high-salt diet augmented the expression of p47phox in the tubules that were induced to be structurally dilated (Fig. 7B, marked with an asterisk). The expression of MCP-1 was also enhanced by a high-salt diet in the highly dilated tubules (Fig. 7E). Cells infiltrating into the interstitial

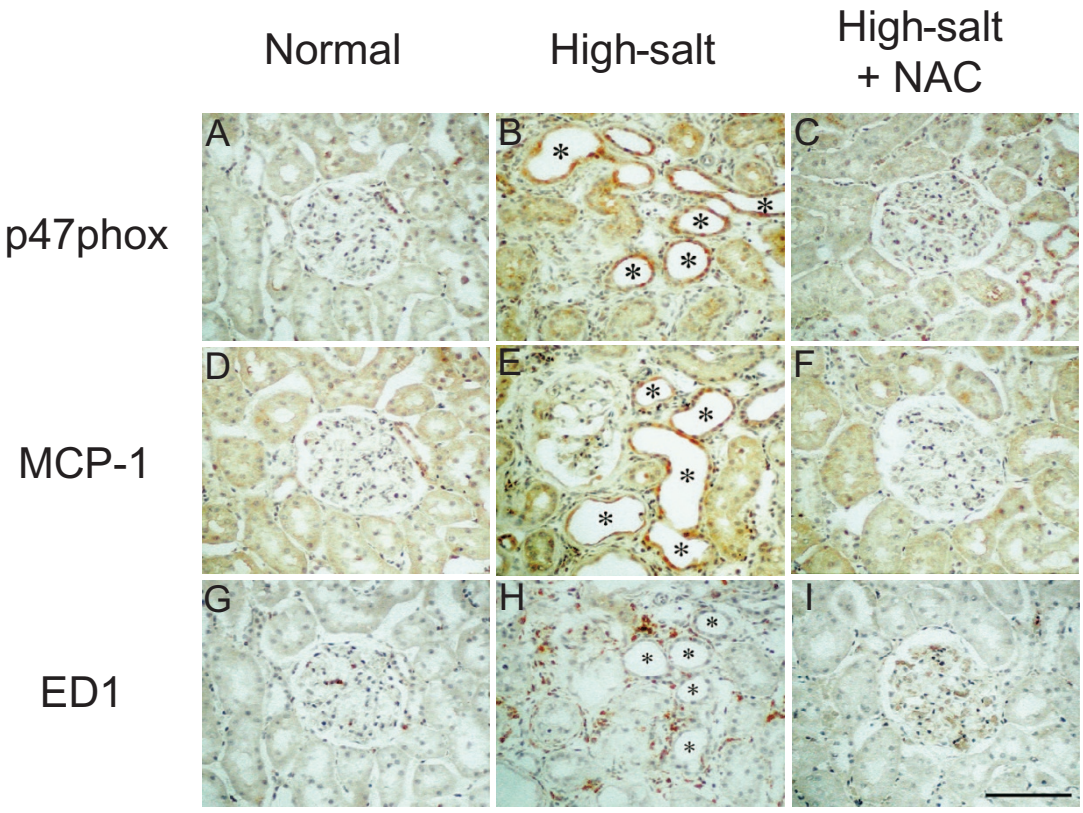

Fig. 7. *Immunohistochemistry of a representative renal cortex section. Staining was performed by a horseradish peroxidase-diaminobenzidine system (brown) with hematoxylin counterstaining (blue nuclei). A–C: For p47phox. Compared with normal-diet rats (A), rats administered a high-salt diet showed a marked increase in the expression of p47phox protein in the structurally highly dilated tubules (asterisk) (B). NAC supplementation blocked the dilation of tubules and thus the expression of p47phox protein (C). D–F: For MCP-1. In high-salt fed DSS rats, prominent staining for MCP-1 was localized to the highly dilated tubules (asterisk) (E). NAC supplementation abrogated the increase of the staining (F). G–I: For ED1. Interstitial infiltration of ED1-positive macrophages was evident in high-salt fed rats (H). Scale bar, 100 μm.*

regions also appeared to be stained for MCP-1 and p47phox; however, this staining was weaker than that in the dilated tubules. NAC supplementation blocked the dilation of tubules and suppressed the augmentation of the expression of p47phox (Fig. 7C) and MCP-1 protein (Fig. 7F). MCP-1 is a chemoattractant for macrophages, which are one of the predominant inflammatory cells found in the interstitium in chronic glomerular diseases (*19*). Macrophage infiltration, as judged by staining using ED1 antibody, was evident in the interstitium in the high-salt group (Fig. 7H), which was counteracted by NAC supplementation (Fig. 7I).

Since albuminuria, which arises from glomerulosclerosis and other glomerular injuries, was associated with tubulointerstitial injury (Fig. 7), we also performed adequate semiquantitative measurements of the tubulointerstitial injury score and glomerulosclerosis score. PAS staining of tissue sections revealed the presence of tubulointerstitial injury, which was characterized by tubular dilatation, thickening of the tubular basement membrane and interstitial cell infiltration. Sclerosis of glomeruli was defined by an increase in PAS-positive materials in the glomerular tufts in high-salt fed DSS rats. Scoring of the renal injury showed that the high-salt diet markedly increased tubulointerstitial injury (Fig. 8A) and glomerulosclerosis (Fig. 8B), and that both these increases were counteracted by NAC supplementation. These results paralleled the results for albuminuria (Fig. 3).

Finally, we examined whether hypotensive treatment with hydralazine reduces proteinuria in the high-salt fed DSS rats. However, when systolic arterial pressure was reduced to 144±3 mmHg at the 4th week by hydralazine, the proteinuria was not improved (478±25 mg/day; $n=4$). NADPH oxidase activity in the renal cortex was not decreased by hydralazine (normal: 1.45±0.15, $n=7$; high-salt: 2.02±0.16, $n=7$; high-salt plus NAC: 1.31±0.11, $n=5$; high-salt plus hydralazine: 2.04±0.14 μmol/min/mg protein, $n=3$). Urinary MCP-1 excretion was not ameliorated by hydralazine (1.9±0.2 ng/day, $n=4$).

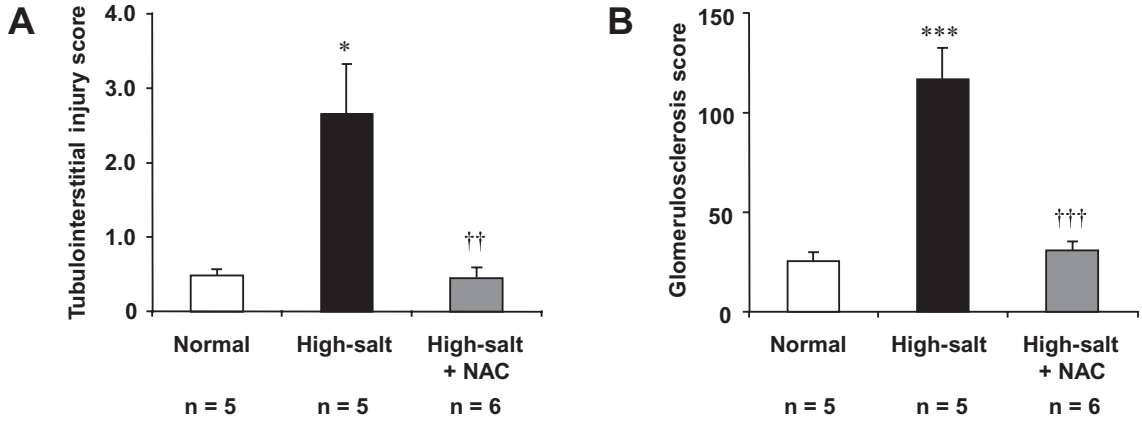

Fig. 8. *Analysis of tubulointerstitial injury (A) and glomerulosclerosis (B) in Dahl salt-sensitive rats. The scores were defined as described in the Methods section. *$p < 0.05$, ***$p < 0.0005$ vs. the normal group. $^{††}p < 0.005$, $^{†††}p < 0.0005$ vs. the high-salt group.*

Discussion

In the present study, a high-salt diet induced glomerular injury and urinary albumin excretion in DSS rats. The MCP-1 expression was intense in the renal tubules, which were structurally dilated probably through the process of reabsorption of the excessive filtered proteins, and thus were assumed to be proximal tubules, although no identification was done. MCP-1 expression was suppressed (Fig. 5) when NAC supplementation completely blocked the increase in the urinary albumin excretion (Fig. 3), suggesting that the excreted albumin induced MCP-1 expression in proximal tubular cells in the DSS rats.

In the case of chronic renal disease progression, the process of reabsorption of filtered proteins activates the proximal tubular cells, leading to tubular injury and interstitial inflammation (*1*). In cultured proximal tubular cells, albumin can stimulate MCP-1 expression (*5*, *6*), although the effects of many other urinary candidate proteins remain to be investigated (*7*). The present study demonstrates that a high-salt diet induces MCP-1 expression in the kidneys of hypertensive DSS rats through a mechanism similar to that of chronic renal diseases, *i.e.*, by increasing albuminuria and proteinuria.

The present results suggest that urinary albumin also induces NADPH oxidase expression in the renal tubular cells. When albuminuria was ameliorated, the expression of NADPH oxidase was down-regulated. The expressions of both p47phox and MCP-1 were strong in the dilated renal tubules of high-salt fed DSS rats, suggesting a relationship—*e.g.*, augmented activation of the NADPH oxidase may have induced renal tubular cells to express MCP-1. Actually, exposure to excess human albumin and immunoglobulin in cultured human proximal tubular cells has been reported to induce the production of H_2O_2, which in turn is responsible for nuclear factor κB (NFκB)–dependent induction of MCP-1 (*6*). Moreover, the urinary excretion pattern of H_2O_2 was the same as that of MCP-1. Namely, high-salt loading increased the amounts of urinary MCP-1 and H_2O_2 excretion per day, but their levels in the 1st week were similar to those in the 4th week (Figs. 4 and 6). These results also imply that the stress induced in the initial week of a high-salt diet is strong enough for maximum induction of H_2O_2 production and MCP-1 expression.

In the present study, a portion of the oxidative stress may have been derived from macrophages. Although many cells can produce superoxide, monocytes/macrophages are likely a significant source of NADPH oxidase–derived superoxide (*20*). Furthermore, MCP-1 activates the respiratory burst of monocytes (*21*, *22*) and induces the expression of tissue factor (*23*), interleukin (IL)-6 and IL-1β (*24*). MCP-1 stimulates collagen synthesis *via* up-regulation of transforming growth factor β_1 expression (*25*) and was capable of stimulating matrix metalloproteinase-1 mRNA expression and synthesis (*26*).

Because NAC supplementation improved hypertension in the present study, it is possible that a decrease in the systemic blood pressure led to the blockage of proteinuria. However, the present study demonstrated that hypotensive treatment with hydralazine did not reduce urinary protein or MCP-1 excretion in the DSS rats. Furthermore, previous studies have reported that hypotensive treatment with the calcium channel blockers TA 3090 (*8*), amlodipine (*27*) and hydralazine (*27*, *28*) did not prevent glomerulosclerosis and proteinuria or albuminuria in DSS rats, because hydralazine (*29*) and amlodipine (*30*, *31*) did not effectively reduce glomerular hypertension compared with systemic hypertension and were thus ineffective in limiting proteinuria. In patients with chronic kidney disease and hypertension, in contrast to the angiotensin II receptor antagonist losartan, amlodipine did not change the amount of proteinuria over a 12-month period (*32*). Bigazzi *et al*. (*33*) showed that a high-salt diet increases

glomerular capillary pressure in salt-sensitive hypertensive patients. They found that albuminuria was higher in salt-sensitive than in salt-resistant patients, and increased with high sodium intake. The albumin excretion rate was significantly correlated with the calculated glomerular capillary pressure. In a study on type II diabetic patients with albuminuria, the resistance of the efferent arterioles and the glomerular pressure were higher than in patients with normoalbuminuria, and the urinary excretion of albumin was correlated with the glomerular pressure but not with the systemic pressure (*34*). It has also been reported that antioxidant treatment normalizes diabetes-induced renal dysfunctions such as albuminuria (*35*). We previously examined the effect of nitric oxide synthase substrate L-arginine in the same DSS model as used in the present study (*10*). The increase in nitric oxide induced by supplementation of L-arginine decreased oxidative stress, proteinuria and systemic blood pressure, probably because nitric oxide can ameliorate glomerular hypertension by scavenging superoxide rapidly. NAC also acts as a scavenger of reactive oxygen species. By raising intracellular levels of cysteine, a precursor of glutathione (GSH), NAC can also help replenish the depleted glutathione stores, leading to maximum activation of glutathione peroxidase for scavenging H_2O_2 (*36*), prevention of damage by scavenging free radical, and blocking activation of NFκB (*37*). The molecular target of NAC-mediated blockade of NFκB activation has not been identified. During hypoxia (*38*) and stimulation by tumor necrosis factor-α (*39*), NFκB is translocated to the nucleus. In these previous studies, NAC did not prevent these translocations, but it did abrogate NFκB binding to DNA. Hypertonic sodium chloride has been shown to activate NFκB in renal medullary interstitial cells (*40*) and to increase reactive oxygen species in human embryonic kidney 293 cells, with the latter effect being reduced by NAC (*41*). Tojo *et al*. (*9*) reported hydrogen peroxide production in the glomeruli of DSS rats fed a diet containing 8% NaCl for 12 weeks. Taken together, these studies suggest that a high-salt diet causes oxidative stress, which induces glomerular hypertension and proteinuria, and NAC can prevent the oxidative stress, glomerular hypertension and thus proteinuria through its antioxidative effect, but not by a decrease in the systemic blood pressure. Long-term administration of NAC to humans is well-tolerated and has been widely used in clinics (*42*). In contrast, cysteine treatment is not readily used *in vivo* for increasing glutathione levels because of its reported neuronal toxicity (*43*). In the chronic renal failure model, NAC attenuates the progression of renal failure, including glomerulosclerosis and proteinuria (*44*).

The current results demonstrate that a high-salt diet induced albumin and protein excretion from glomerular lesions in the DSS rats. The process of reabsorption of filtered proteins activated the renal tubular cells, leading to tubular injury and interstitial inflammation, much as in the progression of chronic renal disease. The reabsorption induced NADPH oxidase expression and H_2O_2 production in the dilated tubules, which led to MCP-1 expression and macrophage infiltration, because elimination of albuminuria and proteinuria after NAC supplementation was effective.

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