Effects of Dietary Salt Restriction on Renal Progression and Interstitial Fibrosis in Adriamycin Nephrosis

Joon-Sung Park    Sua Kim    Chor Ho Jo    Il Hwan Oh    Gheun-Ho Kim

Department of Internal Medicine, Hanyang University College of Medicine, Seoul, Korea

Key words
Fibrosis • Dietary NaCl • Doxorubicin • NF-kappa B • NADPH oxidase

Abstract

Background/Aims: Although high salt intake is thought to accelerate renal progression in proteinuric kidney disease, it is not known whether strict dietary salt restriction could delay renal inflammation and interstitial fibrosis. Here, we sought to answer this question in a rat model of adriamycin-induced nephrotic syndrome. Methods: Adriamycin was administered via the femoral vein in a single bolus (7.5 mg/kg), and the rats were put on a sodium-deficient rodent diet. Rats with intact kidneys were studied for 5 weeks (experiment 1), and uninephrectomized rats were studied for 6 weeks (experiment 2). Results: In experiment 1, restricting salt intake improved renal tubulointerstitial histopathology in adriamycin-treated rats. Immunohistochemical and immunoblot results additionally showed that restricting dietary salt lowered adriamycin-induced expression of osteopontin, collagen III, and fibronectin. In experiment 2, salt restriction improved adriamycin-induced azotemia, although it did not affect proteinuria or blood pressure. Dietary salt restriction also reduced adriamycin-induced infiltration of ED1-positive cells and the upregulated expression of osteopontin and α-SMA. Masson’s trichrome and Sirius red staining revealed that salt restriction slowed adriamycin-induced progression of renal interstitial fibrosis. Finally, qPCR revealed that adriamycin-induced expression of TNF-α, IκB-α, gp91phox, p47phox, and p67phox mRNA was blocked by salt restriction. Conclusion: Our findings demonstrate that strict dietary salt restriction delays the progress of renal inflammation and fibrosis in proteinuric kidney disease, most likely via relieving the reactive oxygen species-mediated NF-κB activation.

J-S Park and S Kim contributed equally to this work.

Gheun-Ho Kim MD, PhD
Department of Internal Medicine, Hanyang University College of Medicine
222 Wangsimni-ro, Seongdong-gu, Seoul, 133-792 (South Korea)
Tel. +82 2 2290 8318, Fax +82 2 2298 9183, E-Mail kimgh@hanyang.ac.kr
Introduction

High dietary intake of salt (NaCl) in patients with chronic kidney disease (CKD) can increase blood pressure and attenuate the antihypertensive and antiproteinuric effects of medications designed to inhibit the renin–angiotensin system [1]. Even in the absence of hypervolemia or arterial hypertension, high salt intake may increase the production of transforming growth factor-β (TGF-β) and lead to renal fibrosis [2, 3]. It may also stimulate NADPH oxidases and increase the production of reactive oxygen species (ROS), resulting in enhanced NF-κB activation and the subsequent production of proinflammatory cytokines [4, 5].

Thus, dietary salt restriction may be advantageous to patients with kidney disease. Previous experimental and clinical studies showed that restricting salt intake reduced proteinuria, delayed the progression of glomerular sclerosis, and preserved kidney function [6-10]. However, these studies excluded cases with heavy proteinuria, and their follow-up was short-term. Our own research showed that dietary salt restriction could potentially reduce tubulointerstitial injury in puromycin aminonucleoside-induced nephrosis and cisplatin nephropathy [11, 12].

On the other hand, radical salt restriction has adverse effects, such as hypovolemia [13]. In addition, contrary to what might be expected, salt restriction appears to upregulate the renin–angiotensin system (RAS) and increase the levels of angiotensin II and NADPH oxidase, which may cause the production of ROS [14]. In patients with CKD, the benefits of reducing salt intake may not be universal [15]. It is also essential to investigate whether low salt intake has long-term benefits in severe glomerulopathy.

Heavy proteinuria is a marker of kidney disease progression. It is often associated with tubulointerstitial injury, which leads to renal fibrosis. Filtered albumin and other proteins accumulate inside proximal tubular epithelial cells (PTECs) and activate multiple signaling pathways [16, 17]. Adriamycin® (ADR, doxorubicin hydrochloride) nephrosis (AN) is a representative animal model of nephrotic syndrome, pathologically characterized by extensive tubular injury, interstitial inflammation, and renal fibrosis; it has been used frequently to study the relationship between glomerular protein ultrafiltration and interstitial fibrogenesis [18]. Using this model, we investigated whether proteinuric kidney disease could be ameliorated by restricting dietary salt. In parallel, we explored the pathways involved in interstitial fibrosis in AN with and without dietary salt restriction.

Materials and Methods

Animal experiments

Pathogen-free, male Sprague-Dawley rats weighing 190–210 g (Orient Bio Inc., Seongnam, Korea) were used in two separate animal experiments: rats with intact kidneys in experiment 1, and uninephrectomized rats in experiment 2. In each experiment, the rats were randomly divided into three groups: vehicle-treated normal salt-fed control animals (vehicle-NS), adriamycin-treated normal salt-fed animals (ADR-NS), and adriamycin-treated low salt-fed animals (ADR-LS). All rats were fed a slurry of the basal diet (18 g/200 g BW) and deionized water (9 mL/200 g BW). The basal diet consisted of a commercially available synthetic rat chow with no added NaCl (DYET #113763 Sodium Deficient AIN-93G Purified Rodent Diet, Dyets Inc., Bethlehem, PA), to restrict the intake of dietary salt in the ADR-LS groups. To provide normal salt levels (0.5%) in the diets consumed by vehicle-NS and ADR-NS rats, NaCl was added to the basal diet. Adriamycin (doxorubicin hydrochloride; Ildong Pharmaceutical, Seoul, Korea) was administered via the femoral vein as a single bolus (7.5 mg/kg), and experiments 1 and 2 were continued for five and six weeks, respectively. Systolic blood pressure was measured using a tail-cuff method (P-98A, Softron; Tokyo, Japan). The experimental protocol was approved by the Institutional Animal Care and Use Committee of Hanyang University.
Renal histopathology

Renal tissue samples were fixed in 4% neutral-buffered formalin and embedded in paraffin. Paraffin sections (4 μm thick) were stained with hematoxylin and periodic acid-Schiff reagent (PAS), Masson’s trichrome, and Sirius red. Tubulointerstitial injury was scored semiquantitatively on PAS-stained tissues, with a ×20 objective, as described previously [19]. Areas of tubulointerstitial fibrosis were quantified on Masson’s trichrome- and Sirius red-stained kidney sections. Fifteen photographs per animal of randomly selected cortical areas were taken at ×200 magnification using a digital inverted microscope (DMI4000B; Leica Microsystems, Wetzlar, Germany). The photographs were scanned to JPEG files with a resolution of 200 pixels/inch. The total positively-stained area in each image was quantified using Leica Application Suite software (Leica Microsystems).

Immunohistochemistry

Immunohistochemical staining to detect ED1, osteopontin, collagen III, α-SMA, and vimentin was performed on formalin-fixed, paraffin-embedded sections using the microwave antigen retrieval method [20]. The primary antibodies were mouse monoclonal anti-rat ED1 (Serotec, Oxford, UK), mouse monoclonal anti-rat osteopontin (MPIIIIB10, the Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), goat polyclonal anti-human collagen III (Sigma, Saint Louis, MO), mouse monoclonal anti-α-SMA (Sigma), and mouse monoclonal anti-human vimentin (Novocastra Laboratories Ltd., Newcastle, UK). Osteopontin, collagen III, α-SMA, and vimentin expression levels were quantified by measuring each immunostained area with a computerized image analyzer (Leica Application Suite): all fields encountered while tracing a serpentine course in a kidney section were assessed under a ×20 objective. A point-counting technique was used to calculate the number of ED1-positive interstitial cells/macrophages in at least 20 consecutive high-power fields.

Immunoblot analysis

Manually dissected slices of whole kidney were homogenized in a buffer containing 250 mM sucrose, 10 mM triethanolamine, 1 μg/mL leupeptin, and 0.1 mg/mL phenylmethylsulfonyl fluoride titrated to pH 7.6. Coomassie-stained “loading gels” were prepared in order to assess the accuracy of protein loading before immunoblotting [12]. Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using 12% gels for fibronectin and GAPDH. For immunoblotting, the proteins were transferred electrophoretically from unstained gels to nitrocellulose membranes (Bio-Rad, Hercules, CA). After blocking with 5% skim milk in PBS-T (80 mM Na₂HPO₄, 20 mM NaH₂PO₄, 100 mM NaCl, 0.1% Tween-20, pH 7.5) for 1 hr, the membranes were probed overnight at 4°C with the respective primary antibodies: rabbit polyclonal anti-human fibronectin (Sigma) and rabbit monoclonal anti-human GAPDH (Cell Signaling Technology, Beverly, MA). The secondary antibody was goat anti-rabbit IgG conjugated to horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA). The sites of antibody-antigen reaction were viewed using enhanced chemiluminescence (GenDEPOT, Barker, TX), and the band densities on the immunoblots were quantified by densitometry using a laser scanner and Quantity One software (Basic version 4.6.9, Bio-Rad).

qPCR analysis

Total RNA was isolated from the rat kidney cortex with TRizol® Reagent (Life Technologies, Carlsbad, CA). RNA was quantified by spectrophotometry, and cDNA synthesis was performed on 3 μg of RNA with SuperScript® III Reverse Transcriptase (Life Technologies). For qPCR, 100 ng of cDNA served as a template for PCR amplification using the Brilliant SYBR green QPCR master mix, according to the manufacturer’s instructions (FastStart DNA Master SYBR Green I; Roche Molecular Biochemicals, Mannheim, Germany). A serial dilution (1 ng - fg/μL) of cDNA was used as a template to generate a standard curve. Nested primers were used to amplify the standard and the kidney cDNA samples (table 1). The standard and the unknown samples were amplified in duplicate in 96-well plates. The thermal profile of the LightCycler® Instrument (Roche Molecular Biochemicals) was optimized with an initial denaturation for 10 minutes at 95°C, and 45 amplification cycles, each consisting of 10 seconds at 95°C, 10 seconds at 60°C, and 10 seconds at 72°C. The comparative Ct method was used to determine the relative amounts of target-mRNA levels, expressed for each sample as a percentage of GAPDH mRNA levels. Ct ratios were analyzed using the LightCycler® Software (Version 4.05). Specificity was verified by a postrun melting-curve analysis [21].
Statistics

Values are presented as means ± SE. Differences among the groups were analyzed using the Kruskal-Wallis test. The Mann-Whitney U-test was used for post-hoc comparisons between groups (Statview software; Abacus Concepts, Berkeley, CA). *P values less than 0.05 were considered statistically significant.

Results

Experiment 1: Effects of dietary salt restriction on AN in rats with intact kidneys

AN was induced by a single intravenous injection (7.5 mg/kg) of adriamycin, and the rats were investigated for five weeks. Table 2 presents the urine and blood plasma results at the end of the five-week period. The adriamycin-treated normal salt-fed (ADR-NS) rats and the adriamycin-treated low salt-fed (ADR-LS) rats gained less body weight than the vehicle-treated normal salt-fed controls (vehicle-NS). Urinary sodium excretion was notably reduced in the ADR-LS rats, reflecting the restricted intake. Adriamycin treatment induced significant proteinuria in both ADR-NS and ADR-LS rats, and to a similar extent. Consistent with this, plasma albumin and total protein levels were significantly reduced in both groups of rats. Despite heavy proteinuria, plasma creatinine and creatinine clearance were not significantly affected by either adriamycin treatment or dietary salt restriction (table 2).

By contrast, renal histopathology differed significantly between all groups. A semiquantitative assessment of tubulointerstitial damage in PAS-stained cortical sections revealed significantly more damage in the ADR-NS group (mean injury score: 3.3 ± 0.2) than...
NS rats, adriamycin treatment significantly upregulated the expression of osteopontin and collagen III in the damaged tubules and the interstitium, respectively. Quantitative image analysis showed that these increases were significantly ameliorated by dietary salt restriction in the ADR-LS rats (fig. 2b).

Along with collagen III, the expression of another major extracellular matrix protein, fibronectin, was altered by both adriamycin and dietary salt restriction. Immunoblotting assays of whole kidney homogenates revealed that the increased fibronectin expression in ADR-NS (5.2 ± 0.8 fold greater than vehicle-NS, \( P < 0.05 \)) was significantly downregulated in the ADR-LS group (3.1 ± 0.6 fold greater, \( P < 0.05 \); fig. 3).

**Experiment 2: Effects of dietary salt restriction on AN in uninephrectomized rats**

To investigate salt restriction in a renal mass reduction model, adriamycin treatment was administered after the rats underwent unilateral right nephrectomy. The rats were then studied for six weeks. Table 3 presents the urine and plasma results at the end of the six-week study. Interestingly, adriamycin treatment was found to increase urine volume. The extent of heavy proteinuria was comparable in the ADR-NS and ADR-LS rats, although urinary sodium excretion was lower in the latter group. Systolic blood pressure was similar in all groups (table 3).
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ADR-LS (1.7 ± 0.7 mg/dL) and ADR-NS groups (3.3 ± 0.8 mg/dL, \( P < 0.05 \)). Consistent with this, creatinine clearance was lower in the ADR-NS group and notably higher in the ADR-LS group (table 3).

The results of Masson's trichrome and Sirius red staining of renal cortex sections were compatible with the above findings (fig. 5). Compared with vehicle-NS, the area of interstitial fibrosis was substantially larger in ADR-NS, and significantly less affected in ADR-LS. Levels of fibronectin were found to be altered by both adriamycin treatment and dietary salt

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Vehicle-NS (n=5)</th>
<th>ADR-NS (n=6)</th>
<th>ADR-LS (n=8)</th>
<th>( P ) value*</th>
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</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>495 ± 9</td>
<td>270 ± 18b</td>
<td>276 ± 7b</td>
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<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>140 ± 15</td>
<td>151 ± 10</td>
<td>152 ± 8</td>
<td>0.717</td>
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<tr>
<td>Urine volume (mL/day/100 g BW)</td>
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<td>10.2 ± 1.4b</td>
<td>7.9 ± 0.5b</td>
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<td>Urine Na⁺ (µEq/day/100 g BW)</td>
<td>397 ± 21</td>
<td>401 ± 57</td>
<td>55 ± 9b</td>
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<tr>
<td>Proteinuria (mg/day/100 g BW)</td>
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<td>244 ± 22b</td>
<td>243 ± 24b</td>
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<tr>
<td>BUN (mg/dL)</td>
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<td>153 ± 41b</td>
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<tr>
<td>Cr (mg/dL)</td>
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<tr>
<td>Cr clearance (µL/min/100 g BW)</td>
<td>0.63 ± 0.03</td>
<td>0.07 ± 0.2b</td>
<td>0.22 ± 0.05d</td>
<td>0.002</td>
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</tbody>
</table>

*Values are mean ± SE. Vehicle-NS, vehicle-treated normal salt-fed controls; ADR-NS, adriamycin-treated normal salt-fed rats; ADR-LS, adriamycin-treated low salt-fed rats; BUN, blood urea nitrogen; Cr, creatinine. *A Kruskal-Wallis test. bP<0.05 vs. Vehicle-NS in a Mann-Whitney U-test. *P<0.05 vs. ADR-NS in a Mann-Whitney U-test; **P<0.05, vs. ADR-NS in a Mann-Whitney U-test.

Fig. 3. Fibronectin in adriamycin nephrotic rats with intact kidneys (experiment 1). (a) Immunoblot of whole kidney homogenates reacted with an antibody to fibronectin (FBN). Each lane was loaded with a protein sample from a different rat. (b) Densitometric analysis. Vehicle-NS, vehicle-treated normal salt-fed controls; ADR-NS, adriamycin-treated normal salt-fed rats; ADR-LS, adriamycin-treated low salt-fed rats. *P<0.05 vs. vehicle-NS; *P<0.05 vs. ADR-NS in a Mann-Whitney U-test.

In contrast with experiment 1, levels of blood urea nitrogen and plasma creatinine increased after adriamycin treatment. As shown in fig. 4, an increase in plasma creatinine was observed from week 2 onwards. By week 6, there was a significant difference in plasma creatinine between the

Fig. 4. Changes in serum creatinine levels in uninephrectomized, adriamycin nephrotic rats (experiment 2), measured at weeks 2, 4, and 6 of experiment 2. Vehicle-NS, vehicle-treated normal salt-fed controls (n=5); ADR-NS, adriamycin-treated normal salt-fed rats (n=6); ADR-LS, adriamycin-treated low salt-fed rats (n=8). *P<0.05 vs. vehicle-NS; *P<0.05 vs. ADR-NS in a Mann-Whitney U-test.
restriction, and in an immunoblot analysis of whole kidney homogenates, AN-upregulated expression of fibronectin (ADR-NS: 8.8 ± 0.8 fold greater than vehicle-NS, \( P < 0.05 \)) was significantly reduced by dietary salt restriction (ADR-LS: 5.8 ± 1.0 fold greater, \( P < 0.05 \)).

As shown in fig. 6, immunohistochemical staining for ED1 pointed to macrophage infiltration in the renal cortex. Adriamycin treatment resulted in a dramatic increase in the number of ED1-positive cells, but restricting salt intake ameliorated this effect. In addition, osteopontin and \( \alpha \)-smooth muscle actin (\( \alpha \)-SMA) responded similarly to adriamycin treatment and to dietary salt restriction (fig. 7). However, the increase in vimentin in ADR-NS was not significantly affected by the salt restriction.

The results of quantitative polymerase chain reaction (qPCR) analysis are summarized in fig. 8. Compared with Vehicle-NS, ADR-NS had higher mRNA expression levels of TGF-\( \beta \), TNF-\( \alpha \), IkB-\( \alpha \), gp91\textsuperscript{phox}, p47\textsuperscript{phox} and p67\textsuperscript{phox}. Compared with ADR-NS, ADR-LS had lower mRNA expression levels of TNF-\( \alpha \), IkB-\( \alpha \), gp91\textsuperscript{phox}, p47\textsuperscript{phox} and p67\textsuperscript{phox}.

**Discussion**

This study demonstrates that, in adriamycin-induced nephrosis, dietary salt restriction can have beneficial effects on both the structure and function of the kidney. Not only azotemia,
The expression of collagen III and fibronectin, two major extracellular matrix proteins that contribute to renal fibrosis, was significantly altered by adriamycin and salt restriction in experiment 1. The presence of interstitial inflammation was confirmed through a semiquantitative assessment of tubulointerstitial injury, changes in peritubular infiltration of ED1-positive cells, and osteopontin expression in the kidney. Previous studies showed that, osteopontin, bound to surface integrins, was associated with a number of intracellular signaling pathways, including phosphoinositide-3-kinase/Akt-dependent NF-κB activation [22], and also with recruitment of inflammatory cells, such as macrophages, T cells and natural killer cells [23-25]. When these are considered together with our findings (i.e., changes in expression of TNF-α and 1κB-α mRNAs), it can be concluded that dietary salt restriction plays a role in the interactions between osteopontin and the NF-κB-activated cascades that is independent of proteinuria, and may attenuate renal inflammation. It was suggested previously that TNF-α is but also renal inflammation and fibrosis, were improved as a result of dietary salt restriction. These changes were associated with a partial reversal of TNF-α, 1κB-α, and NADPH oxidase activation, although not with changes to proteinuria and systolic blood pressure.

We performed experiments with and without uninephrectomy. Although heavy proteinuria was uniformly induced by adriamycin in both experiments, azotemia occurred only in the nephrectomized rats. Accordingly, the effect of dietary salt restriction on interstitial fibrosis was more evident in experiment 2. In addition, the expression of collagen III and fibronectin, two major extracellular matrix proteins that contribute to renal fibrosis, was significantly altered by adriamycin and salt restriction in experiment 1.

The presence of interstitial inflammation was confirmed through a semiquantitative assessment of tubulointerstitial injury, changes in peritubular infiltration of ED1-positive cells, and osteopontin expression in the kidney. Previous studies showed that, osteopontin, bound to surface integrins, was associated with a number of intracellular signaling pathways, including phosphoinositide-3-kinase/Akt-dependent NF-κB activation [22], and also with recruitment of inflammatory cells, such as macrophages, T cells and natural killer cells [23-25]. When these are considered together with our findings (i.e., changes in expression of TNF-α and 1κB-α mRNAs), it can be concluded that dietary salt restriction plays a role in the interactions between osteopontin and the NF-κB-activated cascades that is independent of proteinuria, and may attenuate renal inflammation. It was suggested previously that TNF-α is
an important mediator between PTECs and inflammatory immune cells in the pathogenesis of proteinuric kidney disease [26]. Consistent with a previous report [27], we observed that inhibition of activated TNF-α and NF-κB was associated with a reduction in tubulointerstitial injury in proteinuric rats.

By qPCR, we demonstrated that expression of gp91phox, p47phox, and p67phox, key components of NADPH oxidase, was dramatically upregulated by adriamycin treatment, and that this effect was countered by dietary salt restriction. Previous studies reported that adriamycin [28] and high salt intake [29] stimulated ROS generation, and we found that dietary salt restriction reduced oxidative stress in nephrotic rats. This antioxidant effect could explain the reduced interstitial inflammation and delayed renal fibrosis in proteinuric kidney disease, and it may have been achieved via inhibition of the ROS-mediated activation of NF-κB.

In line with previous studies [30, 31], we found that renal TGF-β expression was upregulated in nephrotic rats. However, in our hands this effect could not be ameliorated by restricting dietary salt, and we believe that TGF-β-independent mechanisms may be involved in the effects observed as a result of salt restriction in nephrosis. Although the induction of α-SMA may be mediated by TGF-β [32], restricting dietary salt was found to reverse the adriamycin-induced upregulation of α-SMA.

There are known adverse effects of restricting dietary salt. Extreme salt deprivation may stimulate the RAS, leading to increased levels of angiotensin II and oxidative stress via NADPH oxidase [14, 33]. However, these downstream effects of stimulation of the RAS were not confirmed in our experiments. Recently, Shao et al. demonstrated that RAS activation due to a low-salt diet did not increase intratubular angiotensinogen and angiotensin II levels in rat kidneys [34]. Another study showed that restricting dietary salt could reduce kidney damage in rats fed a high-fructose diet [35]. This beneficial effect was accompanied by anti-inflammatory responses, although not by systemic hemodynamic improvements. Similarly, we found that blood pressure and proteinuria were not associated with the improvement of azotemia and renal fibrosis.

Conclusion

We demonstrate that dietary salt restriction can ameliorate renal insufficiency and interstitial fibrosis in rats with adriamycin-induced nephrosis. These beneficial effects are accompanied by a reduction in the renal inflammatory response and oxidative stress, suggesting that non-hemodynamic mechanisms are at play. Dietary salt modification may be an important therapeutic option that could delay the progression of proteinuric kidney disease.

Disclosure Statement

None declared.

Acknowledgments

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References


