Blood pressure versus direct mineralocorticoid effects on kidney inflammation and fibrosis in DOCA-salt hypertension

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Abstract

Objective. We examined the contribution of high blood pressure versus direct mineralocorticoid effects to the progression of kidney inflammation and fibrosis in established experimental deoxycorticosterone-acetate (DOCA)-salt hypertension.

Methods. Male Sprague-Dawley rats underwent unilateral nephrectomy and received subcutaneous DOCA pellets as well as 1% NaCl for drinking. After 4 weeks of DOCA-salt hypertension, rats were either killed (n = 6), or treated with a non-hypotensive dose of spironolactone (n = 7) or triple therapy (hydrochlorothiazide, reserpine and hydralazine, n = 8) to normalize blood pressure or with vehicle (n = 19) for two further weeks. Mean arterial pressure (MAP) was measured intra-arterially. Glomerulosclerosis, interstitial fibrosis, macrophage infiltration and complement deposition were evaluated on kidney sections. Expression of collagens, chemokines and cytokines was measured by real-time PCR.

Results. MAP was elevated in DOCA rats, not affected by spironolactone and normalized by triple therapy. Glomerulosclerosis and interstitial fibrosis of DOCA rats were alleviated by spironolactone and triple therapy. Macrophage infiltration, complement C3 deposition and nitrotyrosine staining in the kidney were significantly reduced by spironolactone as well as triple therapy. The expression of collagens, chemokines and cytokines was measured by real-time PCR.

Conclusion. Direct mineralocorticoid effects as well as high blood pressure per se contribute to inflammation and fibrosis of the kidney. Oxidative stress may mediate the direct mineralocorticoid effects on kidney inflammation.
Materials and methods

DOCA-salt hypertension

Rats were housed in a room maintained at 22 ± 2°C, exposed to a 12-h dark/light cycle. All procedures performed on animals were done in accordance with guidelines of the American Physiological Society and were approved by the local government authorities. DOCA-salt hypertension was induced as described previously [17,18]. Briefly, 6-week-old male Sprague-Dawley rats (Charles River, Sulzfeld, Germany) of 180–220 g body weight first underwent left unilateral nephrectomy. After 2 weeks of recovery, 21-day-release DOCA pellets containing 50 mg DOCA (Innovative Research of America, Sarasota, FL, USA) were implanted subcutaneously by incision of the right flank under ether anaesthesia. A second pellet was implanted 21 days later. The animals had free access to 1% saline (10 g NaCl/L). Normotensive control rats (UNX, N = 12) were uninephrectomized and received 1% saline.

Treatments

After 4 weeks of DOCA-salt treatment, hypertensive animals were divided in four groups: six rats were killed to evaluate the degree of renal injury after 4 weeks of hypertension; eight rats received triple therapy with hydrochlorothiazide, reserpine and hydralazine, added to the saline bottles for drinking (DOCA + Triple); seven animals received spironolactone, 100 mg per kg body weight per day, dissolved in sesame oil, by daily gavage (DOCA + Spiro); and nineteen animals were left untreated (DOCA + Vehicle), nine of which received daily gavage of sesame oil.

Measurement of blood pressure and urinary protein excretion

Systolic blood pressure was measured weekly by a tail cuff method under light ether anaesthesia as described previously [19]. On the day of killing, animals were equipped with a femoral catheter under isoflurane anaesthesia, and intraarterial blood pressure was measured in conscious rats 4 h after anaesthesia. Two days before killing, urine was collected for 24 h, using metabolic cages, for measurement of proteinuria (Bio-Rad Protein Assay, Bio-Rad Laboratories, Munich, Germany).

Kidney inflammation and fibrosis

Macrophages/monocytes were counted after staining for the rat macrophage/monocyte marker ED-1 as described previously [17,20]. Interstitial ED-1 positive cells were counted in 20 medium-power (magnification 250×) cortical views per section and expressed as cells per medium-power field. Intraglomerular ED-1 positive cells were counted in all glomeruli of a given section (150–300) and expressed as cells per glomerular cross section. The degree of interstitial fibrosis was determined by evaluation of collagen I staining [17,20]. Immunohistochemistry for collagen I was performed with a rabbit polyclonal antibody to collagen I (Biogenesis, Poole, England), as described previously [17,20]. Computer-based integration of stained areas was performed in 10 low-power views per kidney section. Nitrotyrosine staining as a parameter of oxidative stress was performed using a polyclonal rabbit antibody (Upstate biotechnology, Lake Placid, NY, USA). Staining for complement factor C3 was done with a goat polyclonal antiserum (Cappel, Aurora, OH, USA). Interstitial nitrotyrosine and C3 staining was evaluated by computer-based integration. The number of glomeruli staining positive for C3 was counted. Osteopontin immunoreactivity was evaluated by counting positive points on a point grid after staining with a rabbit polyclonal antibody to osteopontin as described [17]. Glomerulosclerosis was evaluated in kidney sections stained with peroxidase-Schiff’s (PAS) reagent. A score 0–4, based on the sclerotic area of the glomerulus, was used [17]. At least 100 glomeruli per section were evaluated, and the glomerulosclerosis index is given as the mean score per animal.

Gene expression of chemokines, cytokines and adhesion molecules

Total RNA was extracted using the single-step method of Chomczynski [21] with the TRI reagent (Molecular Research Center Inc., Cincinnati, OH, USA). First-strand cDNA was synthesized with TaqMan reverse transcription reagents (Applied Biosystems, Darmstadt, Germany) using random hexamers as primers. Reactions without Multiscribe reverse transcriptase were used as negative controls for genomic DNA contamination. PCR was performed with an ABI PRISM 7000 Sequence Detector System and TaqMan or SYBR Green Universal PCR Master Mix (Applied Biosystems), as described previously [20]. All samples were run in triplicates. The relative amount of the specific mRNA was normalized with respect to 18S rRNA. For the detection of connective tissue growth factor, the primers 5'-TGTGCACCTGCACAAAGATGT-3' and 5'-GTTACACGGACCCACCG-3' were used. For the detection of PAI-1, the primers 5'-GGTACACGGAGCCACCG-3' and 5'-TGTTAGGCAGTTCCAGGGA-3' were used. For detection of TIMP-2, the primers 5'-GCTGACGTGTGAGGAAAGA-3' and 5'-GCACAAATAGTCACAGAGGGTAT-3' and the probe paraffin, 3-µm sections were cut with a Leitz SM 2000 R microtome (Leica Instruments, Nussloch, Germany).
5′-TCTCCTCCGCTTCCCTGCATTTAGA-3′ were used. Previously described oligonucleotides were used for the detection of transforming growth factor beta-1 (TGF-β1) [22], intercellular adhesion molecule-1 (ICAM-1) [20], vascular cell adhesion molecule-1 (VCAM-1) [20], osteopontin (OPN) [23], macrophage chemoattractant protein-1 (MCP-1) [24], collagens I and III [25], TIMP-1 [26] and 18S rRNA [27].

Analysis of data

Analysis of variance (ANOVA), followed by the post hoc Bonferroni test, was used to test significance of differences between groups. A P-value < 0.05 was considered significant. The procedures were carried out using the SPSS software (release 11.5, SPSS Inc., Chicago, IL, USA). Values are displayed as mean ± standard error of the mean.

Results

Systolic blood pressure, as measured by a tail cuff method, was not different between groups before DOCA treatment. Blood pressure rose during DOCA-salt administration, and decreased with further treatment during the last 2 weeks only in the DOCA + Triple group (data not shown). Mean arterial blood pressure in conscious animals is shown in Figure 1. Blood pressure was almost normalized by triple therapy but not significantly affected by spironolactone, compared to untreated DOCA-salt rats. Serum potassium was low in DOCA-salt rats after 6 weeks, compared to UNX (2.9 ± 0.1 versus 3.7 ± 0.1 mmol/L, respectively, \( P = 0.002 \)). Potassium was further reduced by triple therapy (2.3 ± 0.1 mmol/L, \( P = 0.075 \)) but increased by spironolactone (4.2 ± 0.5 mmol/L, \( P < 0.001 \)).

Urinary protein excretion was elevated after 4 weeks of DOCA-salt, and increased further to very high levels in the following 2 weeks (Figure 1). Protein excretion was lowered by triple therapy but not by spironolactone. Body weight was not significantly different between groups \( (P = 0.83 \text{ by ANOVA, Table 1}) \). The relative left ventricular weight was grossly increased by DOCA. Triple therapy but not spironolactone lowered left ventricular weight (Table 1). Both treatments did not significantly lower the markedly elevated relative kidney weight (Table 1). Kidney function, as assessed by serum creatinine and creatinine clearance, decreased progressively in DOCA-salt hypertensive rats (Table 2). Both treatments exhibited non-significant trends towards improved kidney function (Table 2).

Table 1. Body and organ weights.

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>UNX</th>
<th>DOCA 4 weeks</th>
<th>DOCA 6 weeks</th>
<th>DOCA + Triple</th>
<th>DOCA + Spirono</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of rats</td>
<td>12</td>
<td>6</td>
<td>19</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>436 ± 13</td>
<td>346 ± 16*</td>
<td>365 ± 10*</td>
<td>383 ± 11</td>
<td>395 ± 16</td>
</tr>
<tr>
<td>Left ventricle weight/body weight ratio (mg/g)</td>
<td>1.84 ± 0.04</td>
<td>3.12 ± 0.30*</td>
<td>3.10 ± 0.08*</td>
<td>2.67 ± 0.51*</td>
<td>3.11 ± 0.17*</td>
</tr>
<tr>
<td>Right kidney weight/body weight ratio (mg/g)</td>
<td>4.41 ± 0.16</td>
<td>8.15 ± 1.07*</td>
<td>10.70 ± 0.60*</td>
<td>9.73 ± 0.49*</td>
<td>9.07 ± 0.73*</td>
</tr>
</tbody>
</table>

Data are means ± standard errors of the means.

\( ^* P < 0.05 \text{ versus UNX.} \)

\( ^1 P < 0.05 \text{ versus DOCA + Vehicle.} \)

Table 2. Serum creatinine and creatinine clearance.

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>UNX</th>
<th>DOCA 4 weeks</th>
<th>DOCA 6 weeks</th>
<th>DOCA + Triple</th>
<th>DOCA + Spirono</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of rats</td>
<td>12</td>
<td>6</td>
<td>19</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>Serum creatinine (mg/dl)</td>
<td>0.29 ± 0.01</td>
<td>0.36 ± 0.04</td>
<td>0.47 ± 0.02</td>
<td>0.31 ± 0.02</td>
<td>0.41 ± 0.06</td>
</tr>
<tr>
<td>Creatinine clearance (ml/min)</td>
<td>3.85 ± 0.27</td>
<td>3.06 ± 0.50</td>
<td>1.76 ± 0.15*</td>
<td>2.58 ± 0.26*</td>
<td>2.39 ± 0.34*</td>
</tr>
</tbody>
</table>

Data are means ± standard errors of the means.

\( ^* P < 0.05 \text{ versus UNX.} \)
Inflammation in DOCA-salt hypertension

Glomerulosclerosis was present after 4 weeks of DOCA-salt hypertension, and progressed further during the following 2 weeks (Figure 2). Despite the different effects on blood pressure, left ventricular weight and protein excretion, both triple therapy and spironolactone significantly improved glomerulosclerosis and interstitial fibrosis (Figure 2). The effects on glomerulosclerosis were due to a reduction of the number of glomeruli exhibiting the most severe forms of sclerosis. In untreated DOCA rats, 17.5 ± 2.6% of glomeruli showed global sclerosis (score 4). This percentage was reduced to 3.1 ± 1.5% by triple therapy (P = 0.013), and to 4.2 ± 1.9% by spironolactone (P = 0.033), respectively.

Macrophage infiltration of the kidney that was prominent in DOCA-salt rats, as reported previously [17], was markedly alleviated by triple therapy as well as spironolactone (Figure 3). Glomerular macrophage counts were also decreased in spironolactone-treated DOCA-salt rats if compared with 4-week DOCA salt animals (P = 0.017), indicating some degree of regression of inflammation (Figure 3b). A similar trend (P = 0.077) was present in triple-therapy-treated DOCA-salt rats. The deposition of complement C3 was increased in DOCA-salt rats and decreased by spironolactone as well as by triple therapy (Figure 4). A similar pattern was observed for nitrotyrosine staining as a marker of oxidative stress (Figure 5).

We investigated the gene expression of several adhesion molecules and chemoattractants that might contribute to macrophage infiltration. The expression of all factors examined—ICAM-1, VCAM-1, MCP-1 and OPN—was significantly increased by DOCA-salt, and significantly reduced by triple therapy (Table 3). However, none of the mediators was decreased by spironolactone. For OPN protein, quantification of OPN immunohistochemistry (Figure 6) confirmed the RT-PCR findings. Only the expression of MCP-1 exhibited a trend towards lower levels in the DOCA + Spirono group that did not reach statistical significance (Table 3). A similar pattern was observed with regard to collagens I and III as well as the profibrotic mediators PAI-1, TGF-β1 and CTGF (Table 3).
Fig. 4. (A) Examples of immunofluorescence of kidney sections for complement C3 (bright fluorescence). The quantification of C3 is shown for glomeruli (B) and for the cortical interstitium (C). *$P < 0.05$ versus normotensive UNX controls. †$P < 0.05$ versus hypertensive, DOCA-salt-treated rats. Data are mean ± standard error.

Discussion

We examined the effects of blood pressure lowering versus mineralocorticoid receptor blockade on kidney inflammation and fibrosis in DOCA-salt hypertensive rats. Treatments were started after 4 weeks of DOCA-salt administration when high blood pressure and some degree of renal damage were already established. This experimental protocol may limit the potential effects of the treatments, compared to preventive studies in which treatments are started with the induction of hypertension. However, a delayed antihypertensive treatment to inhibit the progression of kidney disease more closely resembles the situation usually encountered in patients. Blood pressure lowering by a triple therapy consisting of a diuretic, a vasodilator and a centrally acting sympathetic agent was compared with the mineralocorticoid receptor antagonist spironolactone at a dose that did not lower blood pressure. Both treatments ameliorated kidney inflammation and fibrosis to a similar degree.

The beneficial effects of blood pressure lowering by triple therapy are easy to interpret. In uninephrectomized DOCA-salt animals, a reduction of systemic arterial pressure will lead to reduced glomerular capillary pressure [28] that in turn will decrease glomerular filtration of proteins, as observed by us. The reduction of tubular protein load will then presumably limit tubulointerstitial inflammation [29]. The net result of less glomerular capillary hypertension, and less proteinuria, will be an amelioration of glomerulosclerosis and interstitial fibrosis, which we observed. Considering the degree of kidney injury already present at the onset of antihypertensive treatment, and the continued administration of DOCA and salt loading, the pronounced effect of triple therapy on all mediators of inflammation is impressive. These data confirm the important role of high blood pressure for target organ damage in mineralocorticoid-salt hypertension [12,15,16].

However, spironolactone was as effective as triple therapy in preventing glomerulosclerosis and interstitial fibrosis. This fact is more difficult to explain, particularly in the absence of an effect on blood pressure. In this context,
We do not think, however, that such an effect could explain our findings. First, this non-genomic vascular action could not be antagonized by spironolactone [30]. Second, the absence of an effect on urinary protein excretion in our study also argues against a marked lowering of glomerular capillary pressure by spironolactone. Blood pressure lowering by triple therapy did clearly reduce proteinuria while spironolactone did not. This observation points to different mechanisms of protection by spironolactone versus triple therapy, respectively. We did not detect a decrease of blood pressure by spironolactone, neither by weekly tail cuff measurements nor by intraarterial recordings from conscious rats at the end of the study. Further, the degree of left ventricular hypertrophy was not affected by spironolactone. While we cannot exclude a small effect on blood pressure, we are convinced that we did not overlook an effect of a magnitude comparable to that exerted by triple therapy.

While the protective effect of spironolactone on the kidney could not be related to decreased blood pressure or decreased proteinuria, it was clearly related to amelioration of macrophage infiltration, complement deposition and oxidative stress. Similar findings were recently reported by Fiebeler et al. [14] in a transgenic rat model of hypertension. We did not perform experiments designed to test for a causal relationship. Raji et al. [31] reported that complement factors are necessary for the development of DOCA-salt induced glomerulosclerosis in mice, supporting the notion that kidney inflammation induces sclerosis. Therefore, we speculate that a reduction of inflammation by mineralocorticoid receptor blockade may explain the beneficial effects on fibrosis [32]. How does spironolactone reduce macrophage infiltration? We investigated the gene expression of several chemoattractants and adhesion molecules that are often considered as potential mediators of the inflammatory effects of aldosterone [9,17,33,34]. To our surprise, all the factors tested were induced by DOCA-salt hypertension but were mostly dependent on high blood pressure and not affected by spironolactone. These data confirm previous reports that mechanical force can induce adhesion molecules [35], OPN [36] and MCP-1 [37]. The same considerations appear to apply to the profibrotic cytokines TGF-β1 [38] and CTGF [39]. The expression of PAI-1, collagen I and

### Table 3. Gene expression of markers of inflammation and fibrosis.

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>UNX</th>
<th>DOCA ± Vehicle</th>
<th>DOCA ± Triple</th>
<th>DOCA ± Spirono</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCP-1 (fold induction)</td>
<td>1.00 ± 0.21</td>
<td>29.05 ± 6.32*</td>
<td>4.13 ± 1.05†</td>
<td>16.97 ± 3.16*</td>
</tr>
<tr>
<td>Osteopontin (fold induction)</td>
<td>1.00 ± 0.38</td>
<td>50.88 ± 9.71*</td>
<td>10.00 ± 2.35†</td>
<td>44.12 ± 13.82*</td>
</tr>
<tr>
<td>ICAM-1 (fold induction)</td>
<td>1.00 ± 0.38</td>
<td>23.85 ± 3.85*</td>
<td>8.46 ± 3.54†</td>
<td>28.46 ± 6.92*</td>
</tr>
<tr>
<td>VCAM-1 (fold induction)</td>
<td>1.00 ± 0.36</td>
<td>6.98 ± 1.32*</td>
<td>2.66 ± 0.77†</td>
<td>8.19 ± 2.69*</td>
</tr>
<tr>
<td>TGF-β (fold induction)</td>
<td>1.00 ± 0.30</td>
<td>12.10 ± 1.90*</td>
<td>3.60 ± 0.97†</td>
<td>14.20 ± 6.30*</td>
</tr>
<tr>
<td>CTGF (fold induction)</td>
<td>1.00 ± 0.21</td>
<td>4.61 ± 0.74*</td>
<td>1.62 ± 0.43†</td>
<td>3.44 ± 0.63*</td>
</tr>
<tr>
<td>Collagen I (fold induction)</td>
<td>1.00 ± 0.25</td>
<td>16.68 ± 6.37*</td>
<td>2.27 ± 0.62†</td>
<td>9.52 ± 2.73</td>
</tr>
<tr>
<td>Collagen III (fold induction)</td>
<td>1.00 ± 0.24</td>
<td>10.47 ± 3.68*</td>
<td>2.51 ± 1.03</td>
<td>6.39 ± 1.67</td>
</tr>
<tr>
<td>PAI-1 (fold induction)</td>
<td>1.00 ± 0.42</td>
<td>13.67 ± 3.67*</td>
<td>2.45 ± 0.53†</td>
<td>8.67 ± 2.64*</td>
</tr>
<tr>
<td>TIMP-1 (fold induction)</td>
<td>1.00 ± 0.27</td>
<td>16.08 ± 4.25*</td>
<td>3.18 ± 0.57†</td>
<td>9.09 ± 2.22</td>
</tr>
<tr>
<td>TIMP-2 (fold induction)</td>
<td>1.00 ± 0.20</td>
<td>3.33 ± 0.77*</td>
<td>1.43 ± 0.25†</td>
<td>3.24 ± 0.75*</td>
</tr>
</tbody>
</table>

Data are means ± standard errors of the means.

*P < 0.05 versus UNX.

†P < 0.05 versus DOCA ± Vehicle.
collagen III was ameliorated by spironolactone in previous studies [40], but we detected only a trend towards lower expression that did not reach significance. In contrast, blood pressure lowering reduced the expression of all these mediators significantly. Thus, our findings underscore the important role of high blood pressure per se for the induction of inflammatory and fibrotic events leading to target organ damage. The effects of spironolactone on macrophage infiltration may be mediated directly by oxidative stress [41] as mineralocorticoid receptor stimulation has been shown to increase the production of reactive oxygen species in glomerular mesangial cells [42] and podocytes [43]. Alternatively, complement deposition [31] or a direct effect of mineralocorticoid receptors on monocytes/macrophages may play a role, as suggested by others [44].

In summary, our results emphasize the importance of blood pressure lowering for the amelioration of kidney inflammation and fibrosis. High blood pressure was the main factor leading to the upregulation of chemokines, adhesion molecules and profibrotic cytokines. However, a direct effect of the mineralocorticoid receptor that could be blocked by spironolactone contributed importantly to glomerular and tubulointerstitial inflammation, independent from blood pressure. Finally, both factors—high blood pressure per se, and direct mineralocorticoid effects—contributed importantly to glomerular and interstitial fibrosis.

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Conflict of interest statement. Drs Schmieder, Veelken and Hilgers have served on advisory boards, and have received grant support for other studies, speaker’s fees, and travel grants from several pharmaceutical companies relating to this study. Speaker’ s fees, and travel grants from several pharmaceutical companies served on advisory boards, and have received grant support for other studies, speaker’s fees, and travel grants from several pharmaceutical companies.

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