Aldosterone/salt induces renal inflammation and fibrosis in hypertensive rats

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Aldosterone/salt induces renal inflammation and fibrosis in hypertensive rats.

Background. We evaluated the role of aldosterone as a mediator of renal inflammation and fibrosis in a rat model of aldosterone/salt hypertension using the selective aldosterone blocker, eplerenone.

Methods. Unnephrectomized, Sprague-Dawley rats were given 1% NaCl (salt) to drink and randomized to receive treatment for 28 days: vehicle infusion (control); 0.75 µg/hour aldosterone subcutaneous infusion; or aldosterone infusion + 100 mg/kg/day oral dose of eplerenone. Blood pressure and urinary albumin were measured and kidneys were evaluated histologically. Renal injury, inflammation, and fibrosis were assessed by immunohistochemistry, in situ hybridization, and reverse transcription-polymerase chain reaction (RT-PCR).

Results. Aldosterone/salt induced severe hypertension compared to controls (220 ± 4 mm Hg vs. 131 ± 4 mm Hg, P < 0.05), which was partially attenuated by eplerenone (179 ± 4 mm Hg, P < 0.05). In aldosterone/salt treated rats, renal histopathologic evaluation revealed severe vascular and glomerular sclerosis, fibrinoid necrosis and thrombosis, interstitial leukocyte infiltration, and tubular damage and regeneration. Aldosterone/salt increased circulating osteopontin (925.0 ± 80.2 ng/mL vs. 53.6 ± 6.3 ng/mL) and albuminuria (75.8 ± 10.9 mg/24 hours vs. 13.2 ± 3.0 mg/24 hours) compared to controls and increased expression of proinflammatory molecules. Treatment with eplerenone reduced systemic osteopontin (58.3 ± 4.2 ng/mL), albuminuria (41.5 ± 7.2 mg/24 hours), and proinflammatory gene expression: osteopontin (OPN), monocyte chemoattractant protein-1 (MCP-1), interleukin-6 (IL-6), and interleukin-1β (IL-1β).

Conclusion. These findings indicate that aldosterone/salt-induced renal injury and fibrosis has inflammatory components involving macrophage infiltration and cytokine up-regulation. Attenuation of renal damage and inflammation by eplerenone supports the protective effects of aldosterone blockade in hypertensive renal disease.

Key words: aldosterone, renal, inflammation, eplerenone, cytokines.

In the kidney, aldosterone mediates salt and water homeostasis by binding to the mineralocorticoid receptor (MR), which is expressed in renal epithelial cells. However, the MR is also present in non epithelial tissues such as in vascular smooth muscle cells, cardiomyocytes, neurons [1], and mononuclear leukocytes [2]. Activation of the MR by aldosterone contributes to kidney damage in experimental models of hypertension. Indeed, administration of aldosterone blockers or aldosterone ablation by adrenalectomy attenuates renal injury in hypertensive rats independent of blood pressure reduction [3, 4].

Aldosterone/salt–induced hypertension has been used for many years as a model of renal disease. The renal damage that develops in this model is characterized histopathologically by severe glomerular injury with vascular fibrinoid necrosis and thrombotic microangiopathy [5], leading to renal fibrosis. Renal lesions are commonly accompanied by a marked inflammatory response, primarily involving mononuclear cells [5]. However, the molecular mechanisms mediating the renal inflammatory changes that occur in response to aldosterone/salt treatment as well as cellular responses mediating the vascular and glomerular damage have not been studied in detail. Recently, Luft [6] and Mervaala et al [7] have characterized some of the inflammatory changes associated with the development of renal injury in models of angiotensin II–induced hypertension. In these studies, angiotensin II was proposed as the major factor responsible for monocyte recruitment and vascular inflammatory changes in the kidney.

In the present study, we evaluated the potential role of aldosterone as a mediator of the cellular and molecular changes leading to renal inflammation and injury. Specifically, the effects of the selective aldosterone blocker, eplerenone [8], on renal inflammation and injury were evaluated in the aldosterone/salt hypertensive rat model. In addition, we studied the cellular localization of some of the inflammatory cytokines involved. Our results indicate that aldosterone/salt–induced renal vascular injury...
and fibrosis are associated with leukocyte infiltration and increased expression of the proinflammatory cytokines osteopontin (OPN), monocyte chemottractant protein-1 (MCP-1), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6), suggesting that inflammation is an important contributor to renal damage in this model.

The role of aldosterone in aldosterone/salt–induced hypertension and end organ damage was evaluated using the novel, selective aldosterone blocker, eplerenone. We provide evidence that aldosterone blockade attenuates renal vascular injury and reduces cytokine expression and subsequent inflammation. These data indicate for the first time that aldosterone, in addition to angiotensin II, plays a pivotal role in renal vascular damage in aldosterone/salt–induced hypertension in part by modulation of inflammatory processes.

METHODS

Animals

The present study was conducted in male Sprague-Dawley rats (250 to 270 g, N = 24), obtained from Harlan Sprague-Dawley Industries (Indianapolis, IN, USA). All experimental procedures received approval by the Institutional Laboratory Animal Care and Use Committees of Pharmacia Corporation. All animals were housed in a room lighted 12 hours per day at an ambient temperature of 22 ± 1°C. Animals were allowed 1 week to adjust after arrival and had free access to rodent diet and tap water until the initiation of the experiment.

Surgical procedure

Laparotomy was performed in rats anesthetized with 1% to 2% isoflurane (AErrane; Baxter, Inc., Deerfield, IL, USA). Following left uninephrectomy, a radiotelemetry implant (Data Sciences, Inc., St. Paul, MN, USA) was inserted in the abdominal aorta approximately 2 to 3 mm above the bifurcation of the iliac arteries for continuous monitoring of blood pressure. The abdominal wall was then sutured and postoperative pain was controlled with a single dose of 0.1 to 0.5 mg/kg subcutaneous injection of buphrenorphine (Rickett & Colman Pharmaceuticals, Inc., Richmond, VA, USA).

Aldosterone and eplerenone treatment

At the time of surgery, an Alzet 2004 osmotic minipump (Alza Corp., Palo Alto, CA, USA) containing either vehicle (9% ethanol/87% propylene glycol/4% H2O) or 2.9 mg/mL d-aldosterone (Sigma Chemical Company, St. Louis, MO, USA) was inserted subcutaneously between the shoulder blades. The dose of aldosterone administered was 0.75 µg/hour.

Eplerenone was administered in the chow (Purina 5002 rodent diet + eplerenone; Research Diets, Inc., New Brunswick, NJ, USA) at a concentration of 1.2 mg eplerenone/g (~1200 ppm) of chow resulting in an approximate dose of 100 mg/kg/day. This dose and route of administration were determined to result in optimal pharmacokinetic characteristics for effective in vivo inhibition of MR in the rat (abstract; Rocha R, Hypertension 38:479, 2001). Previous analytic work in our laboratories has demonstrated the stability of eplerenone in this chow, as well as the homogeneity obtained after preparation. Briefly, pelleted eplerenone chow samples were ground into a fine powder, and in triplicate, 1 g samples were placed into a 125 mL flask with 25 mL of sample diluent [acetonitrile/H2O (60/40) (vol/vol)] and placed onto a 250 rpm shaker for 30 minutes. Samples were then filtered (0.45 µm) and concentration tested using chromatography. Percent recovery was calculated using eplerenone LOQ standard and eplerenone chow samples. Recovery was ~100% (eplerenone chow ppm = 1320).

After recovery from surgery, animals were placed in one of the following 28-day treatment protocols: (1) control (vehicle/normal chow/1% NaCl drinking water) (N = 7), (2) aldosterone infusion (aldosterone/normal chow/1% NaCl drinking water) (N = 9), and (3) aldosterone infusion + eplerenone (aldosterone/eplerenone chow/1% NaCl drinking water) (N = 8).

Blood pressure analysis

Arterial blood pressure was monitored with a radio telemetry system using the Dataquest™ A.R.T™ Version 2.1-Gold software (Data Sciences, Inc., St. Paul, MN, USA). Reported values represent the average of all data points collected over a 24-hour period. Blood pressure was recorded for 10 seconds every 15 minutes throughout the duration of the study. The 24-hour period used was from 6:00 a.m. to 6:00 a.m.

Sacrifice and tissue collection

Animals were anesthetized with pentobarbital (65 mg/kg intraperitoneally), (Sigma Chemical Company) and exsanguinated. Blood samples were centrifuged and plasma was collected and stored at −80°C. Following exsanguination, the right kidney from each rat was removed, rinsed in cold saline, and blotted dry. Kidneys were immediately sectioned through the short axis into five slices. Three slices were fixed in periodate-lysine-paraformaldehyde (9.2 mmol/L Na-m-periodate, 75 mmol/L lysine, 0.2 mol/L paraformaldehyde) for 6 hours followed by 70% ethanol for 18 hours and routinely processed and embedded in paraffin. The remaining two sections were immediately dissected on ice, separating medulla from cortex, and immediately snap-frozen in liquid nitrogen. Frozen samples were stored at −80°C for molecular analysis.

Histopathologic analysis

Kidney sections (3 µm) were stained with the periodic acid-Schiff (PAS) method and with hematoxylin and
eosin and examined by light microscopy in a blinded fashion by a pathologist. The following histologic parameters were graded: glomerular injury, arterial injury, interstitial fibrosis, tubular dilation, protein casts, and inflammatory cell infiltrates. A semiquantitative grading system (0 to 4) was utilized as follows: 0 = no damage, 1 = minimal damage with 1 or a few small foci, 2 = mild damage with small to moderately sized foci, 3 = moderate damage with frequent and moderately sized foci, and 4 = severe damage with extensive confluent foci affecting most of the kidney.

**Immunohistochemistry**

Sections (5 μm) were immunostained following standard procedures using primary antibodies for OPN (working dilution 1:100, The University of Iowa, Iowa City, IA, USA), ED1 (working dilution 1:500; Chemicon, Temecula, CA, USA), CD3 (working dilution 1:1000; DAKO, Carpinteria, CA, USA) and vimentin (working dilution 1:100; Novocastra, Burlingame, CA, USA). Briefly, sections were deparaffinized, rehydrated in ethanol, and processed for antigen retrieval (Target Retrieval Solution, DAKO). Positive staining was detected using appropriate biotin-labeled secondary antibodies, horseradish peroxidase-conjugated streptavidin (DAKO), and by incubating the sections in diaminobenzidine (DAKO). Non-specific isotype-matched immunoglobulin G (IgGs) at similar concentrations were used as primary antibodies for negative controls, and tissues known to express these targets were used as positive controls.

**In situ hybridization**

Probe templates for rat OPN, type III collagen, and rat MCP-1 were generated by RT-PCR and labeled by in vitro transcription with 3P-deoxyuridine triphosphate (dUTP) to generate sense and antisense riboprobes (NEN Life Science Products, Boston, MA, USA). The following primers were used: OPN (forward primer, 5’TGG CAC ATT TGT CTT; reverse primer 3’-AGC CCA TCC AGTC), MCP-1 (forward primer, 5’-CCA TCT ACC TGC TGC TAC TCA; reverse primer, 3’-ATC ACA CTA AAC CTA CAC TAC G), type III collagen (forward primer, 5’-TGG CAC ATT TGT CTT; reverse primer, 3’-CTG ACC TAC CCG A). Sections were deparaffinized in xylene, rehydrated in graded ethanol solutions, and fixed in 4% paraformaldehyde for 10 minutes at 4°C. Tissues were then digested with proteinase K (5 mg/mL; 10 minutes, 37°C), washed, and prehybridized in hybridization buffer [50% formamide, 2X standard sodium citrate (SSC), and 10% dextran sulfate] for 2 hours at 42°C following dehydration in ethanol. Tissues were hybridized overnight at 55°C, washed in SSC buffer, and slides were subsequently dehydrated in a graded series of ethanol containing NH4OAc and dried in a vacuum desiccator. Slides were coated with photographic emulsion (Kodak, Rochester, NY, USA) and exposed for 3 and 5 weeks at 4°C prior to development. Developed slides were counterstained with hematoxylin and eosin.

**Plasma and urine assays**

Urinary albumin, plasma OPN, and plasma renin activity were assayed according to standard procedures. Briefly, albumin was determined using the Nephat Albumin enzyme-linked immunosorbent assay (ELISA) kit (Exocell, Philadelphia, PA, USA). Rat OPN was determined using a rat OPN EIA kit (IBL Co., Gunma, Japan). Plasma renin activity was determined by standard radioimmunoassay (RIA) procedures for an estimation of the primary product of renin activity, angiotensin I (NEN Life Science Products).

**RNA isolation**

RNA was extracted from frozen renal cortex using the Totally RNA Isolation Kit (Ambion, Inc., Austin, TX, USA) as previously described [9]. Tissues were crushed, homogenized, and denatured according to manufacturer’s instructions. RNA was further purified by DNase digestion to remove genomic DNA and LiCl precipitation to remove carbohydrates. Briefly, 100 μg of RNA was incubated for 45 minutes at 37°C with 1 unit of DNase (Roche Diagnostics, Indianapolis, IN, USA) and 10 units RNase inhibitor (Applied Biosystems, Foster City, CA, USA) in a buffer containing 40 mmol/L Tris pH 7.8, 6 mmol/L MgCl2, and 10 mmol/L CaCl2. DNase and buffer were removed using RNeasy (Qiagen, Valencia, CA, USA), RNA was precipitated with 7.5 mol/L LiCl/50 mmol/L ethylenediaminetetraacetic acid (EDTA), and incubated overnight at −20°C. All samples were diluted and analyzed spectrophotometrically for concentration and purity and stored at −80°C.

**TaqMan quantitative RT-PCR**

All primers and probes were designed using Primer Express software supplied with the 7700 Sequence Detection System based on known rat sequences and synthesized by Applied Biosystems (Foster City, CA, USA) as previously described [10]. Standard curves were performed to determine the efficiency of each primer/probe set in the TaqMan reaction prior to the analysis of the experimental samples. All target gene results were normalized to the housekeeping gene cyclophilin. Total, purified RNA (200 ng) was added to a RT-PCR reaction mix, which contained the following: 12.5 μL of 2X One-Step PCR Master Mix without uracil-N-glycosylase, 0.625 μL of a 40X MultiScribe and RNase Inhibitor Mix, 0.625 μL of 20 mmol/L forward primer, 0.625 μL of 20 mmol/L reverse primer, 0.5 mL of 5 mmol/L TaqMan probe, and 0.125 mL of DNase/RNase-free water. All samples were analyzed in duplicate. The following protocol was applied to all reactions: 30 minutes at 48°C (re-
Table 1. TaqMan primer-probe sets

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osteopontin (OPN)</td>
<td>CCAGCCACACAAGC</td>
<td>TCAATGCAATAAGC</td>
<td>CAGTCGATGCTCCTGA</td>
</tr>
<tr>
<td></td>
<td>AGACGGT</td>
<td>CAGGGCG</td>
<td></td>
</tr>
<tr>
<td>Monocyte chemoattractant protein (MCP-1)</td>
<td>GCAGGTCTCTGTCA</td>
<td>GGCTGAGACAGCAGTGGAT</td>
<td>CTGGTGTACAGTTCGCTG</td>
</tr>
<tr>
<td></td>
<td>CACTATTGT</td>
<td>CAGTACCTAATTGTC</td>
<td></td>
</tr>
<tr>
<td>Interleukin-6 (IL-6)</td>
<td>AGGAGGGCACTGT</td>
<td>ATCGTGAGGAGCGAG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GATCCTGGAA</td>
<td>GAGCCGGGAGCGAG</td>
<td></td>
</tr>
<tr>
<td>Interleukin-1β (IL-1β)</td>
<td>ATATGTTTCAGGA</td>
<td>TGCATCCTACGCTG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GATCTGAGGAGCG</td>
<td>TGCATCCTACGCTG</td>
<td></td>
</tr>
<tr>
<td>Cyclophilin</td>
<td>AGAGAAATTGGAGG</td>
<td>TTGTTTGTGAGGAG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATGAGAATCTCAT</td>
<td>CCAGGACATGCTCG</td>
<td></td>
</tr>
</tbody>
</table>

All oligonucleotides are written 5’ and 3’. Primers are unlabeled and all probes are labeled at the 5’ end with 6-carboxyfluorescein (6FAM) reporter dye and at the 3’ end with 6-carboxy-N,N’,N’-tetramethylrhodamine (TAMRA) quencher dye.

verse transcription), 10 minutes at 95°C (inactivation of reverse transcriptase), 40 cycles of 15 seconds at 95°C and 1 minute at 60°C (PCR). Data analysis was performed using the Sequence Detection System software from Applied Biosystems. Table 1 contains complete primer and probe sequences for OPN, IL-1β, IL-6, MCP-1, and cyclophilin genes.

Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA). Parametric analysis was performed for systolic blood pressure and biochemical assays data. In all other cases, statistical analysis was performed on the rank transforms of the raw data or ordinal data (nonparametric analysis) to account for any inequality of variance. The alpha = 0.05 level of significance was used for the planned comparisons between the means. The Least Significant Differences (LSD) method was used for planned comparisons between groups. Data were analyzed using PROC GLM in the SAS statistical software package (SAS PC, version 6.12, SAS Institute, Cary, NC, USA). All data are reported as mean ± standard error mean (SEM).

RESULTS

Blood pressure

Systolic blood pressure did not increase in control animals over 28 days (final measurement, 131 ± 4 mm Hg) (Fig. 1). Aldosterone/salt treatment induced a progressive increase in blood pressure over time, resulting in severe hypertension (final measurement, 220 ± 4 mm Hg). Animals receiving eplerenone also developed a progressive increase in blood pressure, although this increase was not as large as in the aldosterone/salt–treated animals (final measurement, 179 ± 4 mm Hg).

Physiologic and biochemical characteristics

All physiologic and biochemical data are presented in Table 2. Body weights were significantly lower in aldosterone/salt–treated animals compared to controls. Eplerenone-treated animals maintained body weights similar to control animals. Urinary albumin was markedly elevated in aldosterone/salt–treated animals compared to control animals and eplerenone significantly reduced aldosterone/salt–induced albuminuria. Plasma renin activity (PRA) was low in all experimental groups (Table 2). Aldosterone infusion significantly elevated plasma OPN levels (925 ± 80.1 ng/mL vs. 53.6 ± 6.3 ng/mL, P < 0.05) compared to controls, whereas eplerenone treated animals had plasma OPN levels that were similar to controls (58.3 ± 4.2 ng/mL vs. 53.6 ± 6.3 ng/mL).
Table 2. Physiologic and biochemical characteristics

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>Control</th>
<th>Aldosterone/ 1% NaCl</th>
<th>Aldosterone/ 1% NaCl + eplerenone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic blood pressure</td>
<td>mm Hg</td>
<td>131 ± 4</td>
<td>220 ± 4a</td>
<td>179 ± 4b</td>
</tr>
<tr>
<td>Body weight</td>
<td>g</td>
<td>380 ± 4</td>
<td>293 ± 9a</td>
<td>388 ± 7</td>
</tr>
<tr>
<td>Plasma renin activity</td>
<td>ng/mL/hour</td>
<td>0.37 ± 0.11</td>
<td>0.89 ± 0.25a</td>
<td>0.06 ± 0.03b</td>
</tr>
<tr>
<td>Plasma osteopontin</td>
<td>ng/mL</td>
<td>53.6 ± 6.3</td>
<td>925.0 ± 80.2b</td>
<td>58.3 ± 4.2b</td>
</tr>
<tr>
<td>Urinary albumin</td>
<td>mg/24 hours</td>
<td>13.2 ± 3.0</td>
<td>75.8 ± 10.9a</td>
<td>41.5 ± 7.2a,b</td>
</tr>
</tbody>
</table>

Eplerenone administered at 100 mg/kg/day. Values represent group mean ± SEM.

aP < 0.05 vs. control; bP < 0.05 vs. aldosterone/1% NaCl

Table 3. Histopathological renal scores

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>Control</th>
<th>Aldosterone/ 1% NaCl</th>
<th>Aldosterone/ 1% NaCl + eplerenone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number affected glomeruli</td>
<td>(0–4)</td>
<td>0.0 ± 0.0</td>
<td>3.4 ± 0.2a</td>
<td>1.5 ± 0.3a,b</td>
</tr>
<tr>
<td>Number arteries affected</td>
<td>(0–4)</td>
<td>0.0 ± 0.0</td>
<td>3.7 ± 0.3a</td>
<td>0.5 ± 0.3a,b</td>
</tr>
<tr>
<td>Interstitial fibrosis</td>
<td>(0–4)</td>
<td>0.2 ± 0.2</td>
<td>2.8 ± 0.3a</td>
<td>1.3 ± 0.3a,b</td>
</tr>
<tr>
<td>Tubular dilation</td>
<td>(0–4)</td>
<td>0.3 ± 0.2</td>
<td>3.2 ± 0.4a</td>
<td>1.3 ± 0.2a,b</td>
</tr>
<tr>
<td>Protein casts</td>
<td>(0–4)</td>
<td>0.3 ± 0.2</td>
<td>3.1 ± 0.4a</td>
<td>1.1 ± 0.1a,b</td>
</tr>
<tr>
<td>Inflammatory cells</td>
<td>(0–4)</td>
<td>0.3 ± 0.2</td>
<td>2.3 ± 0.2</td>
<td>1.1 ± 0.2a,b</td>
</tr>
</tbody>
</table>

Eplerenone administered at 100 mg/kg/day. Values represent group mean ± SEM.

aP < 0.05 vs. control; bP < 0.05 vs. aldosterone/1% NaCl

Renal histopathology

Mean histopathology kidney grades per group are presented in Table 3. Kidneys from control animals were histologically normal (Fig. 2A), while kidneys from aldosterone/salt–treated animals demonstrated severe glomerular and renal vascular injury (Fig. 2B). Renal vascular damage consisted of severe degenerative changes in the arterioles and small arteries characterized by circumferential, transmural fibrinoid necrosis, thrombosis, myointimal proliferation, and adventitial fibrosis resulting in thickening of the vessel walls. Glomerular changes consisted of expansion of glomerular tufts with fibrin deposition, dilation, and thrombosis of glomerular capillaries, and various degrees of mesangiolysis, hemorrhage, and early glomerular sclerosis. Renal vascular and glomerular lesions were accompanied by inflammatory changes with leukocyte infiltration and multifocal interstitial fibrosis. Consistent with the high levels of albuminuria, significant tubular dilation associated with large proteinaceous casts was observed. In animals receiving eplerenone, lesions were markedly reduced compared to aldosterone/salt–treated animals (Fig. 2C).

Immunohistochemical and in situ hybridization analysis

Kidney sections of aldosterone/salt–treated animals contained frequent interstitial inflammatory cell infiltrates in contrast to those of control animals. These inflammatory cell infiltrates consisted primarily of ED1-positive macrophages (Fig. 2E) infiltrating the cortical interstitium as well as areas of necrosis and degeneration, while CD3-positive T cells were observed multifocally in the interstitium (Fig. 2H). ED1- and CD3-positive cells were less frequent in eplerenone–treated animals (Fig. 2F and I). The degree of tubular damage and regeneration was evaluated by vimentin immunohistochemistry. Vimentin-positive tubular epithelial cells were rarely found in control animals (Fig. 2J). In contrast, in aldosterone/salt–treated animals, many tubules were vimentin positive (Fig. 2K), while the density of vimentin-positive tubules was markedly lower in the eplerenone–treated animals (Fig. 2L). The degree of interstitial fibrosis was evaluated using a routine semiquantitative histopathologic examination. Additionally, type III collagen expression was visualized and localized by in situ hybridization. While rarely detected in both control and eplerenone–treated animals (Fig. 2M and O), type III collagen mRNA was easily detected in the cortex of aldosterone/1% NaCl rats (Fig. 2N), indicating fibrosis. By immunohistochemistry and in situ hybridization, OPN expression was limited to scattered medullary tubules in control animals. In contrast, in aldosterone/salt–treated animals, OPN expression was markedly up-regulated throughout the medulla in both proximal and distal cortical tubules, with the most robust up-regulation affecting the damaged and regenerative tubules (Fig. 3A and B). Scattered myointimal cells of arteries and infiltrating macrophages also expressed OPN. However, positive staining for OPN was only occasionally detected in injured glomeruli. Administration of eplerenone dramatically decreased the num-
Fig. 2. Representative photomicrographs from kidneys of control (left column), aldosterone/1% NaCl (middle column), and aldosterone/1% NaCl + eplerenone (right column) rats after 28 days of treatment. Periodic acid-Schiff (PAS) staining. Kidneys from control rats appeared normal (A), while kidneys from aldosterone/1% NaCl animals (B) displayed marked vascular and glomerular damage characterized by arterial fibrinoid necrosis with segmental myointimal proliferation and adventitial fibrosis (black arrow), glomerular mesangiolyis with fibrinoid necrosis and thrombosis (arrowhead), leukocyte infiltration, protein casts, and tubular dilation (white arrows). With eplerenone treatment (C), injuries were
Fig. 3. Representative photomicrographs of osteopontin (OPN) immunohistochemistry and in situ hybridization. OPN was detected in the medulla (loop of Henle, distal nephrons) of control rats but only rarely in the cortex. Following aldosterone/1% NaCl treatment (A and B), OPN expression was markedly up-regulated in the medulla and in both proximal and distal cortical tubules, with the most robust up-regulation affecting the damaged and regenerative tubules (arrows). Scattered myointimal cells of arteries (arrowheads) and infiltrating macrophages also expressed OPN. Aldosterone/1% NaCl rats treated with eplerenone exhibited only limited OPN staining (C and D). (A and C, ×200; B and D, ×200, bright-field microscopy).

The number of cortical and medullary tubules expressing OPN, as well as the intensity of the signals observed by in situ hybridization, indicating the marked overall reduction in renal OPN expression following eplerenone treatment (Fig. 3 C and D). In control animals, MCP-1 mRNA could not be detected by in situ hybridization. In contrast, in aldosterone/salt–treated animals, MCP-1 mRNA was detected multifocally in damaged and regenerative tubules, interstitial macrophages, scattered vascular smooth muscle cells and arteriolar endothelial cells, and glomerular endothelial and epithelial cells (Fig. 4 A and B). Administration of eplerenone markedly reduced MCP-1 expression in the kidney, as MCP-1 mRNA could only be detected in scattered interstitial macrophages (Fig. 4 C and D).

Renal gene expression

Expression of proinflammatory marker genes was quantitatively assessed to examine the potential molecular mediators of aldosterone/salt–induced renal damage.

markedly reduced (A to C, ×100). ED1 and CD3 immunostaining of control, aldosterone/1% NaCl and aldosterone/1% NaCl + eplerenone rats. In contrast to those of control animals (D and G), kidney sections of aldosterone/1% NaCl rats contained frequent ED1-positive macrophages (E) within the interstitium (arrow) and blood vessels (arrowhead), as well as CD3-positive T cells (H; arrow). ED1 and CD3 immunohistochemistry demonstrated a lower number of infiltrating macrophages and T cells in the eplerenone-treated rats (F and I) (D to L, ×100). Vimentin immunohistochemistry. Vimentin-positive tubular epithelial cells were rarely found in control (J) and aldosterone/1% NaCl + eplerenone (L) rats in contrast to aldosterone/1% NaCl rats, where many tubules were vimentin positive (K; arrows) (I to L, ×50). Type III collagen in situ hybridization was used to demonstrate the early fibrosis in aldosterone/1% NaCl rats (N; arrows), while type III collagen mRNAs were rarely detected in both control (M) and aldosterone/1% NaCl + eplerenone (O) rats (M to O, ×50, dark-field microscopy).
Renal expression levels of OPN, MCP-1, IL-1β, and IL-6 were significantly increased following 28 days of aldosterone infusion compared to controls and this elevated expression was significantly attenuated by eplerenone treatment (Fig. 5 A to D).

DISCUSSION

In this study, we investigated the renal cellular and molecular changes associated with aldosterone/salt hypertension in the rat. Aldosterone/salt treatment induced severe hypertension and associated renal vascular injury, glomerulosclerosis, tubular dilation and injury, and interstitial fibrosis. Our results indicate that aldosterone/salt-induced renal injury and fibrosis is associated with leukocyte infiltration and increased expression of various proinflammatory cytokines, including OPN, MCP-1, IL-1β, and IL-6, suggesting that inflammation is an important component of renal damage in this model. In addition, renal injury, leukocyte infiltration and cytokine expression levels were all markedly attenuated by eplerenone, a selective aldosterone blocker, supporting the renoprotective effects of MR antagonism in hypertensive nephropathy. In the present study, the renal inflammatory response and marked vascular and glomerular damage induced by aldosterone/salt treatment are prominent. Although previous studies demonstrate the existence of an inflammatory response prior to the development of myocardial injury in the aldosterone/salt rat, in the absence of a time-course evaluation, a causal relationship between inflammation and renal vascular damage cannot be established in the current study [11].

Historically, renal inflammation has been described as a feature of experimental hypertensive renal injury models. In the mineralocorticoid/salt hypertension model, original studies by Selye [12] described nephrosclerotic lesions associated with proteinuria and inflammatory vascular changes, which he described as “lesions of peri-
reported the up-regulated expression of various pro-inflammatory molecules [MCP-1, vascular endothelial adhesion molecule, intracellular adhesion molecule, tumor necrosis factor-α (TNF-α), IL-1, and OPN] in high renin-driven hypertensive renal injury models where angiotensin II was proposed to be the primary mediator of renal inflammation and injury [7, 16–19].

As a component of the extracellular matrix as well as a circulating adhesion molecule, the chemotactic cytokine, OPN likely plays a role in the renal inflammatory response following aldosterone/salt treatment by contributing to macrophage recruitment and adhesion [20–22]. Renal OPN expression was primarily localized to injured and regenerating tubules throughout the medulla and cortex and was also present in infiltrating macrophages. Elevated OPN expression and localization were associated with the severity of renal damage in aldosterone/salt–treated animals. No significant OPN expression was found in glomeruli except for those in which marked injury and associated macrophage infiltration was present. These findings are consistent with studies in nephropathy models where significant macrophage accumulation and OPN expression also involved damaged tubules [15, 17]. They are also in agreement with previous findings in the DOCA-salt hypertensive rat, which demonstrated marked OPN expression in both damaged glomeruli and tubules [23]. In the present study, circulating OPN levels were significantly elevated in plasma from aldosterone/salt–treated animals compared to control and deoxycorticosterone acetate (DOCA)-salt hypertensive rats. More recently, Beswick et al [13] demonstrated that leukocyte infiltration in this model is associated with reactive oxygen species accumulation and nuclear factor-kappa B (NF-κB) activation [13]. The current study extends these findings indicating that the cytokines OPN, MCP-1, IL-1β, and IL-6 are significantly up-regulated in the kidney of aldosterone/salt hypertensive rats. In addition, we report the presence of a severe cellular inflammatory response involving primarily monocyte/macrophages, but also T-cell lymphocytes in the kidney of aldosterone/salt hypertensive rats. To our knowledge, this study provides the first description of aldosterone/salt–induced renal proinflammatory cytokine expression and elucidation of the role of aldosterone in this process.

The role of inflammatory cytokine activation in aldosterone-induced hypertensive renal injury is not well understood. IL-6 and IL-1β were up-regulated following aldosterone infusion together with the chemotactic and adhesive factors, MCP-1 and OPN. IL-6 has been shown to induce MCP-1 expression in human mesangial cells [14], while IL-1β is known to directly up-regulate OPN expression in renal epithelial cells [15]. However, from the present study, it is not possible to understand fully the relationship between the different cytokines and their individual contributions to renovascular damage in aldosterone/salt hypertensive rats. Previous studies have also
mation and injury may be related to the significant reduction in albuminuria. Indeed, excessive urinary protein has been described as a potential mediator of renal pro-inflammatory gene expression [15, 29, 30].

CONCLUSION

Animals treated with the mineralocorticoid, aldosterone, in the presence of salt developed severe hypertension, renal inflammation and injury, albuminuria, and elevated expression of the proinflammatory molecules OPN, MCP-1, IL-1β and IL-6. Aldosterone-induced renal injury, inflammation, and fibrosis were effectively attenuated by the selective aldosterone blocker, eplerenone. We postulate that aldosterone may, at least in part, contribute to renal inflammation and injury via modulation of cytokine expression, which promotes inflammatory cell infiltration and albuminuria in hypertension-driven renal injury. Thus, the establishment of aldosterone as a pivotal contributor to renal hypertensive disease has become paramount to understanding the etiology of this disease state.

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REFERENCES