Osteopontin deficiency protects against aldosterone-induced inflammation, oxidative stress, and interstitial fibrosis in the kidney

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Osteopontin (OPN) has been characterized as a highly acidic, phosphoprotein component of bone matrix that is also present in organs such as the brain, pancreas, kidney, and the vasculature. OPN is associated with a variety of epithelial and tumor cells and also with macrophages and lymphocytes. In addition to its participation in cell adhesion and migration mediated by its interaction with various integrins and CD44, OPN also acts as a potent chemokine for mononuclear and vascular smooth muscle cells. It has been proposed that OPN participates in various pathophysiological processes including malignancy (4), atherosclerosis (9, 10, 19, 22), wound healing (20), infection and immunity (2, 5), and myocardial remodeling (35). In the kidney, OPN is produced mainly by the distal nephron and is thought to play a role in the renal damage associated with inflammatory glomerulonephritis, obstructive uropathy, and tubulointerstitial disease (39). However, the precise pathophysiological mechanism by which OPN causes these renal changes remains unclear.

Recent studies have suggested that in addition to aldosterone's classic effects on renal sodium transport and water homeostasis, initiated by mineralocorticoid receptors (MRs) in aldosterone-sensitive nephrons, the hormone also has deleterious effects on nonepithelial cells in the kidney. There is also evidence that activation of MRs by aldosterone contributes to kidney damage in experimental models of hypertension (3, 31), while Blasi et al. (3) demonstrated that OPN has a role in the renal inflammatory response and renal damage following aldosterone and high-salt treatment in experimental rats. Recently, we showed that OPN also has a critical role in collagen synthesis and renal fibroblast proliferation induced by aldosterone (11).

It is interesting from a clinical point of view that we showed serum aldosterone levels were an independent determinant of plasma OPN levels in patients with essential hypertension (17). Similarly, there is evidence that patients with hyperaldosteronism have increased plasma OPN levels compared with patients with essential hypertension (12). Taken together, these findings of basic and clinical studies imply that OPN induced by aldosterone may have a practical role in the development of renal injury. The aim of the present study was to obtain direct evidence of the critical role of OPN in aldosterone-mediated renal injury by infusing aldosterone into either wild-type (WT) or OPN knockout mice (OPN−/−). We showed that Aldosterone-deficiency protected against aldosterone-induced inflammation, oxidative stress, and interstitial fibrosis in the kidney.

MATERIALS AND METHODS

Animals and experimental protocol. OPN−/− mice were generated as described previously (30). Eight-week-old male OPN−/− mice (n = 20) and age-matched male C57BL/6 WT mice (n = 30) were used. All the mice were housed in a room with a 12:12-h light-dark cycle, with the room temperature maintained at 24°C. The experimental protocol was approved by the Animal Studies Committee of Ehime University. After a 2-wk training period for blood pressure measurements, the mice had a left uninephrectomy, carried out at week 0 under...
pentobarbital sodium anesthesia (50 mg/kg, intraperitoneal injection). At the same time, either aldosterone (0.15 µg/h, Sigma-Aldrich, St. Louis, MO) or vehicle was infused subcutaneously using an osmotic minipump (Alzet model 2004, Alza). Eplerenone treatment was administered as described previously (n = 10) (28). Beginning at week 0, all the mice were provided with drinking water containing 1% NaCl and administered chow supplemented with eplerenone at 2 mg/g.

Measurements of systolic blood pressure (SBP) and heart rate were performed using the indirect tail-cuff method (MK-2000; Muromachi Kikai, Tokyo, Japan), while 24-h urine samples were collected in metabolic cages. At the end of the study, blood samples were obtained from the inferior vena cava and the kidneys were then removed and weighed. Coronal sections of the kidney were fixed in 10% formalin, followed by embedding in paraffin for histological evaluation. The remainder of the kidney was snap-frozen in liquid nitrogen for mRNA and protein analysis.

Biochemical measurements. Urine albumin and creatinine were measured by ELISA commercial kits (Exocell, Philadelphia, PA). Serum potassium, urinary potassium, and urinary sodium were measured using an automated analyzer (Nagahama LSL, Shiga, Japan), and urinary 8-isoprostane levels were determined using an ELISA kit (Thermo Scientific). Urine albumin and creatinine were measured by ELISA commercial kits (Exocell, Philadelphia, PA). Serum potassium, urinary potassium, and urinary sodium were measured by ELISA commercial kits (Exocell, Philadelphia, PA).

Table 1. Biological parameters in aldosterone-infused mice at 4 wk.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild-Type</th>
<th>Aldosterone-infused mice</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle (n = 15)</td>
<td>Aldosterone (n = 15)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight, g</td>
<td>29.3 ± 0.2</td>
<td>29.4 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Kidney weight/body weight ratio, mg/g</td>
<td>8.5 ± 0.2</td>
<td>10.7 ± 0.3*</td>
<td></td>
</tr>
<tr>
<td>Systolic blood pressure (final), mmHg</td>
<td>98.5 ± 3.1</td>
<td>129.0 ± 2.2*†</td>
<td></td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>730 ± 5.3</td>
<td>729 ± 8.9</td>
<td></td>
</tr>
<tr>
<td>Creatinine, mg/dl</td>
<td>0.12 ± 0.2</td>
<td>0.13 ± 0.1</td>
<td></td>
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<tr>
<td>Serum potassium, meq/l</td>
<td>4.63 ± 0.2</td>
<td>3.27 ± 0.1†</td>
<td></td>
</tr>
<tr>
<td>Urine Na/K ratio</td>
<td>9.05 ± 1.4</td>
<td>3.55 ± 0.3*†</td>
<td></td>
</tr>
<tr>
<td>Urine Na, µeq/day</td>
<td>4,346 ± 432</td>
<td>1,460 ± 220*†</td>
<td></td>
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Values are means ± SE; n = no. of mice. OPN, osteopontin. *P < 0.01 vs. vehicle/wild-type mice, †P < 0.01 vs. vehicle/OPN-/- mice.

DNA isolation and real-time PCR. Total RNA was isolated from the cortical area of the kidneys using ISOGEN (Nippon gene, Tokyo, Japan) according to the protocol provided by the manufacturer. Gene expression was analyzed quantitatively by real-time RT-PCR using fluorescent SYBR green technology (Roche Applied Bioscience, Indianapolis, IN). β-Actin cDNA was amplified and quantitated in each cDNA preparation to normalize the relative amounts of the target genes. The primer sequences were as follows: OPN (forward 5'- CCAACGGCCGAGGTGATAGC-3', reverse 5'-GCCCTTCTGTTGTCCTGTA-3'); type I collagen (forward 5'-ATCTCTGTTGTCGTAACCC-3', reverse 5'-ACCTTCTGGTGGCAGTC-3'); type III collagen (forward 5'-AGGCAACAGTGTCTCCTG-3', reverse 5'-GACCTCTTGCTCCAGTTAGC-3'); type IV collagen (forward 5'-GGCAGTCAACCGACAGCAT-3', reverse 5'-TGTTGACATCAAGAGAAAGA-3'); fibronectin (forward 5'-CGAGTAGCACAGGCCACAACA-3', reverse 5'-CTGGACAGGAGGAAAGA-3'), reverse 5'-CGCTTCTGATGTTACATACGG-3'; p67phox (forward 5'-CTGGCTGGAGACCACGAGCAGA-3', reverse 5'-CTTGGAAGATGGGAAAGG-3'), reverse 5'-CTTGGAAGATGGGAAAGG-3'), reverse 5'-CTTGGAAGATGGGAAAGG-3'), reverse 5'-CTTGGAAGATGGGAAAGG-3'), reverse 5'-CTTGGAAGATGGGAAAGG-3'), reverse 5'-CTTGGAAGATGGGAAAGG-3'), reverse 5'-CTTGGAAGATGGGAAAGG-3'); interleukin-6 (forward 5'-CCAAAGCAGATCTCCATTAG-3', reverse 5'-TTGCTCTAGCCCTTCTTCC-3'); and β-actin (forward 5'-TGTATTGAAGGCTTGTGGTCTT-3', reverse 5'-AGGTGTGCACTTATTTGTTGCTCAA-3').
nary sodium/potassium ratio among any of the groups. In vehicle-infused mice, SBP did not increase during the 4-wk study. Aldosterone infusion increased SBP in the WT and OPN−/− mice to the same extent throughout the period of infusion [aldosterone/WT 129 ± 2.2 mmHg (n = 15); aldosterone/OPN−/− 123 ± 3.1 mmHg (n = 10)]. There was no difference in serum creatinine levels between the WT and OPN−/− mice, while serum potassium levels were lower in aldosterone-infused mice than in vehicle-infused mice. Urinary sodium excretion and urinary sodium/potassium ratio were increased significantly in the two groups administered aldosterone, although there was no difference in these parameters between aldosterone-infused WT and OPN−/− mice.

Effect of OPN on urinary albumin excretion. Baseline urinary albumin excretion adjusted for urinary creatinine excretion was similar in the WT and OPN−/− mice. As shown in Fig. 1A, infusion of aldosterone for 4 wk caused significant increases in urinary albumin excretion in WT mice [aldosterone/WT 132.9 ± 21.0 μg/mg (n = 15)] and vehicle/WT 29.3 ± 4.0 μg/mg (n = 15)). Surprisingly, in OPN−/− mice, urinary albumin excretion was markedly reduced, even with aldosterone infusion [aldosterone/OPN−/− 33.0 μg/mg (n = 10)].

Effect of aldosterone on renal OPN expression. As expected, OPN expression was essentially absent in aldosterone- and vehicle-infused OPN−/− mice. The detection of OPN mRNA in the renal cortex of WT mice was significantly higher in aldosterone-infused mice than in vehicle-infused mice [aldosterone/WT 3.6 ± 0.3-fold (n = 10); vehicle/WT 1.0 ± 0.1-fold (n = 10); Fig. 1B). As shown in Fig. 1C, aldosterone increased the OPN protein level in WT mice (n = 10 for each). Immunohistochemical analysis showed localization of OPN protein expression in medullary tubules in healthy control mice, whereas this expression was upregulated markedly in the proximal tubules of aldosterone-infused WT mice (Fig. 1D). Strong OPN expression was noted in areas with tissue damage, such as dilated tubules.

Effect of eplerenone on OPN expression. Immunohistochemical analysis (Fig. 2A) and Western blot analysis (n = 6 for each, Fig. 2B) both demonstrated that expression of OPN protein was significantly higher in aldosterone-infused WT mice and that this upregulation was suppressed by treatment with the selective MR blocker eplerenone. Interstitial fibrosis, evaluated by Masson’s trichrome staining, and macrophage infiltration, detected by F4/80 staining, were also increased in proximity to OPN-positive tubules in aldosterone-infused WT mice. Eplerenone treatment reduced these changes in aldosterone-infused WT mice. To evaluate whether MR was activated in both strains of mice, we next performed immunohistochemical analysis of MR in the kidney (Fig. 2C) and evaluated Sgk1 gene expression using RT-PCR (data not shown). As shown in Fig. 2C, MR was localized mainly in the nuclei in tubular cells in both aldosterone-infused WT and OPN−/− mice. Aldosterone caused significant increases in Sgk1 gene expression in both strains, although renal Sgk1 expression levels were similar in the two groups. These results suggested that aldosterone induces OPN expression, interstitial fibrosis, and inflammatory infiltration via MR activation.

Effect of OPN on interstitial fibrosis. Figure 3A shows the typical interstitial pathological changes observed at the end of the study period. Interstitial fibrosis was increased significantly after aldosterone treatment in the WT mice. These changes were attenuated by OPN knockdown as shown by semiquantitated analysis of sections stained with Masson’s trichrome (aldosterone/WT 15.3 ± 1.2%; vehicle/WT 2.1 ± 0.3%; Fig. 3F). Importantly, OPN deficiency almost completely abolished this aldosterone-induced interstitial fibrosis (aldosterone/OPN−/− 2.4 ± 1.4%; Fig. 3F).

Effect of OPN on macrophage infiltration, fibroblast proliferation, and epithelial-mesenchymal transition. As shown in Fig. 3, B and C, macrophages or fibroblasts, detected by immunohistochemical staining for F4/80 or FSP-1, were observed rarely within the tubular interstitium of vehicle-infused WT or OPN−/− mice. However, in aldosterone-infused WT mice the interstitial regions showed significant infiltration of both macrophages (aldosterone/WT 3.42 ± 0.30%; vehicle/WT 0.15 ± 0.03%; Fig. 3G) and fibroblasts (aldosterone/WT 1.17 ± 0.09%; vehicle/WT 0.27 ± 0.01%; Fig. 3H). In contrast, F4/80-positive areas were decreased significantly in aldosterone-infused OPN−/− mice (0.37 ± 0.04%). FSP-1-positive areas were also decreased in aldosterone-infused OPN−/− mice (0.31 ± 0.04%). α-SMA is a good marker for active fibroblasts and phenotypic changes of myofibroblasts in renal tubular cells undergoing epithelial-mesenchymal transition (EMT). We therefore performed immunohistochemical analysis of α-SMA and showed that its expression was increased in aldosterone-infused WT mice compared with vehicle-infused WT mice. In contrast, α-SMA expression in aldosterone-infused OPN−/− mice was attenuated significantly compared with aldosterone-infused WT mice (Fig. 3, D and I). Aldosterone also caused significant decreases in the level of the EMT marker E-cadherin in WT mice, with this downregulation being suppressed by OPN deficiency (Fig. 3, E and J). These data indicated that OPN deficiency attenuated aldosterone-induced macrophage recruitment, fibroblast proliferation, and EMT in the kidney. To determine whether OPN deficiency altered aldosterone-induced inflammation, we next measured gene expression of interleukin-6 (Fig. 3K). We showed that aldosterone increased interleukin-6 gene expression in WT mice and, interestingly, that OPN deficiency also decreased this expression.

Effect of OPN on expression of fibrosis-related genes. In WT mice, aldosterone increased renal mRNA expression of type I collagen [aldosterone/WT 3.5 ± 0.3-fold (n = 13); vehicle/WT 1.0 ± 0.1-fold (n = 10); Fig. 4A]; type III collagen [aldosterone/WT 4.2 ± 0.4-fold (n = 13); vehicle/WT 1.1 ± 0.1-fold (n = 10); Fig. 4B]; type IV collagen [aldosterone/WT 1.5 ± 0.1-fold (n = 13); vehicle/WT 0.8 ± 0.3-fold (n = 10); Fig. 4C]; and fibronectin [aldosterone/WT 2.7 ± 0.2-fold (n = 13); vehicle/WT 1.0 ± 0.1-fold (n = 10); Fig. 4D]. In contrast, expression of fibrosis-related genes was reduced in aldosterone-infused OPN−/− mice [type I collagen 1.4 ± 0.2-fold (n = 8); type III collagen 1.5 ± 0.1-fold (n = 8); type IV collagen 1.1 ± 0.1-fold (n = 8); and fibronectin 1.5 ± 0.2-fold (n = 8)].

Role of OPN in oxidative stress. To determine whether OPN deficiency altered aldosterone-induced oxidative stress, we measured mRNA expression of NADPH oxidase subunits containing p47phox (Fig. 5A), p67phox (Fig. 5B), and gp91phox (Fig. 5C). NADPH oxidase is a multicomponent enzyme complex that consists of membrane-bound gp91phox and p22phox and the cytosolic subunits p67phox and p47phox. We found that aldosterone-infused WT mice had increased expression of
p47phox [aldosterone/WT 1.9 ± 0.2-fold (n = 13); vehicle/WT 1.1 ± 0.1-fold (n = 10)]; p67phox [aldosterone/WT 1.8 ± 0.1-fold (n = 13); vehicle/WT 1.1 ± 0.1-fold (n = 10)]; and gp91phox [aldosterone/WT 2.0 ± 0.2-fold (n = 13); vehicle/WT 1.0 ± 0.1-fold (n = 10)]. In contrast, OPN deficiency inhibited the expression of aldosterone-induced NADPH oxidase subunits [p47phox 1.1 ± 0.2-fold (n = 8); p67phox 1.1 ± 0.1-fold (n = 8); and gp91phox 1.2 ± 0.1-fold (n = 8)]. Aldosterone also increased protein expression of p47phox (Fig. 5D), p67phox (Fig. 5E), and gp91phox (Fig. 5F) in WT mice (n = 6 for each). We also performed immunohistochemical staining for p47phox (Fig. 5G) that showed expression of the
protein induced by aldosterone was located mainly in the renal tubules in WT mice. Interestingly, OPN deficiency decreased the protein expression of each NADPH oxidase subunit. To measure the effect of OPN on renal oxidative stress, we measured urinary levels of 8-isoprostane (Fig. 6A). We observed a marked increase in 8-isoprostane excretion in aldosterone-infused WT mice [aldosterone/WT 1.9 ± 0.3 ng/day (n = 15); vehicle/WT 0.2 ± 0.1 ng/day (n = 15)]. In contrast, this aldosterone-induced increase in 8-isoprostane excretion in WT mice was reduced significantly by OPN knockdown [aldosterone/OPN−/− 0.6 ± 0.2 ng/day (n = 10)]. We also found increases in 8-OHdG-positive tubular cells in aldosterone-infused WT mice (Fig. 6, B and C). The 8-OHdG-positive cells per area was greater in aldosterone-infused WT mice than in aldosterone-infused OPN−/− mice.

Podocyte injury. We next evaluated desmin expression, a marker of podocyte injury (Fig. 7, A and C). Aldosterone-infused WT showed increased desmin expression in glomeruli (aldosterone/WT 1.92 ± 0.13%; vehicle/WT 0.30 ± 0.05%). In contrast, OPN deficiency partially suppressed protein expression of desmin after aldosterone infusion (aldosterone/OPN−/− 1.38 ± 0.12%). We also analyzed nephrin expression, a slit diaphragm protein (Fig. 7, B and D). The nephrin staining area was reduced in aldosterone-infused WT mice compared to vehicle-infused WT mice, but this was not significantly different in aldosterone-infused OPN−/− mice.
infused WT mice, which was also partially prevented by OPN deficiency (aldosterone/WT 0.68 ± 0.11%; vehicle/WT 3.57 ± 0.14%; aldosterone/OPN−/− 2.28 ± 0.18%; vehicle/OPN−/− 3.68 ± 0.14%).

**DISCUSSION**

There is increasing evidence that OPN has a pivotal role in the progression of renal fibrosis. Persy et al. (27) demonstrated that OPN deficiency attenuated interstitial fibrosis in the kidney following ischemia-reperfusion injury in mice. Similarly, Yoo et al. (40) showed that interstitial fibrosis in OPN−/− mice with unilateral ureteral obstruction was less severe than in OPN+/+ mice. More recently, Wolak et al. (38) reported that OPN modulated renal tissue fibrosis in angiotensin II-induced renal injury. These findings suggest that upregulation of OPN may be implicated in tubulointerstitial fibrosis in the kidney.

Evidence from studies in animal models also indicates that aldosterone causes renal fibrosis, glomerular injury, and inflammation. In these studies, aldosterone-induced renal fibrosis appeared to be associated with upregulation of inflammatory cytokines, including OPN (3), before the development of renal fibrosis. We reported recently that aldosterone upregulated OPN expression and that OPN-small interference RNA completely blocked aldosterone-induced collagen synthesis and renal fibroblast proliferation in renal fibroblasts (11). Although transcriptional regulation of OPN expression by aldosterone has been demonstrated in renal fibroblasts (11) and mesangial cells (8), the role of inflammatory cytokine activation by OPN in aldosterone-induced renal fibrosis has not been studied in detail. We hypothesized that aldosterone may promote development of renal fibrosis by inducing OPN expression. In the present study, we demonstrated that aldosterone induced renal fibrosis, albuminuria, fibrosis-related gene expression, and oxidative stress. This is the first study to show that these changes were attenuated significantly by OPN knockdown. Taken together, these results indicate that OPN is a key fibrotic factor contributing to the development of renal fibrosis and that suppression of OPN expression prevents the progression of renal fibrosis associated with various types of nephropathy, including aldosterone-induced nephropathy. Interestingly, urinary albumin excretion, an important predictor for end-stage kidney disease, and the renal fibrosis score were inhibited significantly in aldosterone-infused OPN−/− mice.

Fig. 3. A: representative micrographs of Masson’s trichrome-stained renal sections from aldosterone-infused WT mice (a), vehicle-infused WT mice (b), aldosterone-infused OPN−/− mice (c), and vehicle-infused OPN−/− mice (d; n = 10 for each). Scale bar = 100 μm. B: immunohistochemistry of F4/80 in aldosterone-infused WT mice (a), vehicle-infused WT mice (b), aldosterone-infused OPN−/− mice (c), and vehicle-infused OPN−/− mice (d). Scale bar = 100 μm. C: immunohistochemistry of fibroblast-specific protein-1 (FSP-1) in aldosterone-infused WT mice (a), vehicle-infused WT mice (b), aldosterone-infused OPN−/− mice (c), and vehicle-infused OPN−/− mice (d). Scale bar = 100 μm. D: immunohistochemistry of α-smooth muscle actin (SMA) in aldosterone-infused WT mice (a), vehicle-infused WT mice (b), aldosterone-infused OPN−/− mice (c), and vehicle-infused OPN−/− mice (d). Scale bar = 50 μm. E: immunohistochemistry of E-cadherin in aldosterone-infused WT mice (a), vehicle-infused WT mice (b), aldosterone-infused OPN−/− mice (c), and vehicle-infused OPN−/− mice (d). Scale bar = 50 μm. F: bar graph shows semiquantitative analysis of renal fibrotic area (%). G: semiquantitative analysis of F4/80-positive area (%). H: semiquantitative analysis of FSP-1-positive area (%). I: semiquantitative analysis of α-SMA-positive area (%). J: bar graph shows scoring for E-cadherin staining. K: real-time PCR for interleukin-6. Values are means ± SE. *P < 0.001 vs. vehicle/WT group. #P < 0.001 vs. aldosterone/WT group.
Fig. 5. Real-time PCR and Western blotting for p47^phox (A and D), p67^phox (B and E), and gp91^phox (C and F). Values are means ± SE. *P < 0.001 vs. vehicle/WT group. #P < 0.001 vs. aldosterone/WT group. †P < 0.01 vs. aldosterone/WT group. G: immunohistochemistry of p-47^phox in aldosterone-infused WT mice (a), vehicle-infused WT mice (b), aldosterone-infused OPN⁻/⁻ mice (c), and vehicle-infused OPN⁻/⁻ mice (d). Scale bar = 50 μm.
In this study, there were no significant differences in blood pressure levels at baseline or after aldosterone infusion between the WT and OPN\(^{-/-}\) mice. Urinary sodium excretion and urinary sodium/potassium ratio were not different between aldosterone-infused WT and OPN\(^{-/-}\) mice. It may be the reason there was no difference in blood pressure between two groups. In the present study, aldosterone infusion and a high-salt diet for 4 wk induced pathological changes mainly in interstitial lesions but not in glomeruli. These pathophysiological changes did not affect renal glomerular filtration and no difference in serum creatinine between any of the groups.

The mechanisms by which OPN contributes to interstitial fibrosis remain unclear. Inflammation with macrophage infiltration is a key mechanism in the progression of renal fibrosis (16, 32). In accordance with previous studies on aldosterone-induced renal injury (33, 34), the present study showed that aldosterone infusions resulted in significant increases in macrophage infiltration, detected by immunohistochemical staining of the murine macrophage marker F4/80. On the other hand, OPN\(^{-/-}\) mice had markedly decreased macrophage infiltration in the kidney, indicating that OPN is a major macrophage chemoattractant (6). Kelly et al. (15) also demonstrated that OPN upregulation in association with macrophage influx played an important role in tubulointerstitial injury in diabetic nephropathy. In this regard, infiltrated macrophages are known to produce matrix proteins such as type I collagen (36).

Another important observation in our study was that aldosterone-infused OPN\(^{-/-}\) mice had significant decreases in urinary albumin excretion compared with aldosterone-infused WT mice. Exposure of the proximal tubules to leaked protein is thought to trigger tubulointerstitial fibrosis (1, 23). The contribution of podocyte injury to the progression of albuminuria has been indicated. Some previous studies demonstrated that podocytes are a potential target for aldosterone and MR activation. Furthermore, increased expression of OPN in podocytes plays an important role in the development of albuminuria in glomerular disease models (21). We therefore evaluated podocyte injury using immunohistochemical staining for desmin and nephrin. Aldosterone-infused WT mice showed increased desmin expression in glomeruli. In contrast, OPN deficiency partially suppressed protein expression of desmin after aldosterone infusion. The nephrin staining area was reduced in aldosterone-infused WT mice, which was also partially prevented by OPN deficiency. However, in aldosterone-infused...
infused WT mice in our study, OPN was highly expressed in proximal tubules, but not in glomeruli containing podocytes or mesangial cells. As shown in Figs. 1D and 2A, aldosterone can cause severe injury to the proximal tubules, resulting in tubulointerstitial fibrosis and inflammation. This suggests that albuminuria in our models may be due to podocyte abnormalities, as well as tubular defects. OPN deficiency may therefore indirectly protect the filtration of albumin from glomeruli and directly attenuate protein reabsorption damage in proximal tubules. In the present study, OPN was expressed mainly in the proximal tubules, indicating that abnormal protein traffic in proximal tubules may also induce tubulointerstitial damage and OPN expression. However, in our study we could not ascertain whether the proximal tubular changes were induced by aldosterone or were secondary to albuminuria. Further study is therefore required to clarify the precise mechanism of these changes.

EMT is thought to contribute greatly to renal fibrosis, with a large proportion of interstitial fibroblasts originating from renal tubular epithelial cells (13, 25). EMT in epithelial cells is characterized by the disruption of epithelial junctional complexes and subsequent loss of cell polarity. EMT is accompanied by downregulation of epithelial markers such as E-cadherin and zonula occludens-1, and upregulation of mesenchymal markers, including α-SMA and FSP-1. FSP-1 is a fibroblast-specific protein and is expressed by tubular epithelial cells that undergo EMT, a process in which the tubular epithelial cell changes its phenotype and migrates into the interstitium (14). Although we did not observe FSP-1 staining of tubular epithelial cells, aldosterone treatment markedly increased FSP-1-positive staining in the interstitium in WT mice, while OPN deficiency reduced FSP-1-positive cells even following aldosterone infusion. We also showed that aldosterone induced α-SMA expression and loss of E-cadherin in WT mice. α-SMA is the most commonly used indicator of EMT. E-cadherin is an adhesive junctional protein that maintains the structural integrity and polarity of renal epithelial cells, and is a marker of EMT. We demonstrated that OPN deficiency reduced α-SMA expression in the interstitium and suppressed E-cadherin downregulation in proximal tubules. These results suggest that OPN may have a role in the pathogenesis of EMT.

**Fig. 7.** A: semiquantitative analysis of desmin-positive area (%). B: semiquantitative analysis of nephrin-positive area (%). Values are means ± SE. *P < 0.001 vs. vehicle/WT group. †P < 0.001 vs. aldosterone/WT group. ††P < 0.01 vs. aldosterone/WT group. C: immunohistochemistry of desmin in aldosterone-infused WT mice (a), vehicle-infused WT mice (b), aldosterone-infused OPN−/− mice (c), and vehicle-infused OPN−/− mice (d). Scale bar = 50 μm. D: immunohistochemistry of nephrin in aldosterone-infused WT mice (a), vehicle-infused WT mice (b), aldosterone-infused OPN−/− mice (c), and vehicle-infused OPN−/− mice (d). Scale bar = 50 μm.
The process of fibrosis is preceded by inflammation and oxidative stress (41). Oxidative stress is regarded as an important mediator of aldosterone-induced renal damage. Shibata et al. (31) demonstrated that aldosterone-infused rats had increased gene expression of gp91phox, p67phox, and p47phox. Similar findings were reported by Miyata et al. (24), who showed that aldosterone-induced mesangial injury was associated with an increase in oxidative stress, as determined by expression of NADPH oxidase subunits such as p47phox and p67phox. In accordance with previous reports, we showed in this study that expression of NADPH oxidase subunits, the cytosolic subunits p47phox and p67phox, and membrane-bound gp91phox was increased by aldosterone treatment. In previous studies, OPN appeared to involve both pro- and anti-inflammatory actions. Lai et al. (18) demonstrated a proinflammatory action of OPN by showing that OPN deficiency reduced activation by TNF-α of the NADPH oxidase subunits Nox1, Nox2, p47phox, and p67phox in aortic adventitial myofibroblasts. In contrast, Wolak et al. (38) reported an anti-inflammatory action of OPN by showing that the angiotensin II-induced upregulation of the NADPH oxidase subunits, Nox2, Nox4, and gp47phox was increased significantly in OPN−/− mice. In our study, the absence of OPN was associated with significant decreases in expression of aldosterone-induced NADPH oxidase subunits, indicating a proinflammatory and prooxidant action of OPN. Moreover, 8-isoprostane is a product of the oxidizing modification of arachidonic acid and DNA and is considered a useful marker of oxidative stress (26, 29). 8-OHdG is a DNA base-modified product and is the most commonly used marker for evaluating oxidative DNA damage. In the present study, evidence of substantial renal oxidative stress was observed in WT mice following aldosterone infusion, indicated by significantly elevated 8-isoprostane excretion and 8-OHdG expression in tubular cells. In this model, increased excretion and expression of this oxidative metabolite indicated an elevation in renal oxidative stress associated with aldosterone infusion. We observed that deletion of OPN in our mice reduced 8-isoprostane levels and the degree of 8-OHdG expression, even following infusion of aldosterone. These results were interpreted as indicating that the prooxidant and proinflammatory effects of OPN may be involved in aldosterone-induced renal fibrosis.

We found in this study that eplerenone abolished the aldosterone-induced expression of OPN and renal fibrosis evaluated by Masson’s trichrome stain. This finding indicated the critical role of MR signaling in our model. As OPN deficiency and eplerenone treatment had equivalent ability to prevent renal fibrosis in our model, it is possible this preventative effect of eplerenone may be mediated through inhibition of OPN induction. In an in vitro study in rat renal fibroblasts, we showed that aldosterone-induced OPN expression was abolished by the MR antagonist spironolactone (11). These results suggest that aldosterone may regulate renal inflammation and fibrosis through induction of OPN via MR activation.

In conclusion, the present study in experimental mice demonstrated that OPN deficiency protects against aldosterone-induced inflammation, oxidative stress, and interstitial fibrosis in the kidney. Plasma aldosterone is reported to be an independent predictor of accelerated loss of renal function in hypertension-related renal injury (37). Based on our results, OPN may be a key mediator of aldosterone-induced renal fibrosis, thereby exacerbating kidney disease. Inhibition of OPN may therefore provide a potential target for therapeutic intervention aimed at preventing the progression of renal injury.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES


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