

## Aldosterone-induced kidney injury is mediated by NF $\kappa$ B activation

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### Abstract

**Background** Aldosterone induces inflammation and fibrosis in the kidney, while nuclear factor  $\kappa$ B (NF $\kappa$ B) plays key roles in inflammation mediated by various cytokines. Here, we determined the roles of NF $\kappa$ B activation in aldosterone-induced kidney injury.

**Methods** We used unilaterally nephrectomized rats with or without continuous aldosterone infusion and 0.9% saline as drinking water for 3 weeks. IMD-1041, an IKK $\beta$  inhibitor, and spironolactone were orally administered to inhibit NF $\kappa$ B and mineralocorticoid receptor, respectively.

**Results** The aldosterone-infused rats exhibited severe kidney injury, hypertension, and increased expression of pro-inflammatory and fibrotic proteins, osteopontin, fibrinogen, collagen type I, and PAI-1. Western blotting confirmed NF $\kappa$ B activation by aldosterone by the increased amount of p65 in the nuclear fraction of the kidney, and oral IMD-1041 prevented the kidney injury and lessened the increase in pro-inflammatory and fibrotic proteins

without significant changes in blood pressures. In addition, changes in angiotensin-converting enzyme 2 (ACE2), which has been found to act as a protective factor in various kidney injury models, were examined. Immunofluorescence studies revealed the presence of ACE2 in the brush-border membrane of the proximal convoluted tubules and markedly blunted ACE2 staining in aldosterone-infused rats. The decrease in amount of ACE2 protein was confirmed by Western blotting, and IMD-1041 also prevented the decrease in ACE2. The administration of spironolactone also abolished the effects of aldosterone.

**Conclusion** Our results suggest that aldosterone induces kidney injury via activation of NF $\kappa$ B and mineralocorticoid receptor, and that decreased ACE2 expression may play an important role in aldosterone-induced kidney injury.

**Keywords** Aldosterone · Cytokines · Inflammation · Progression of chronic renal failure

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### Introduction

Aldosterone is known to exert both classical and non-classical actions. The classical actions on the kidney are mainly observed in collecting duct cells, where it induces potassium secretion and sodium reabsorption. Aldosterone's non-classical actions cause tissue injury in various organs, including the heart, arteries, and kidney [1]. Both the classical and the non-classical actions of aldosterone are mediated by a specific mineralocorticoid receptor and are inhibited by its antagonists, spironolactone and eplerenone. In collecting duct cells, it has been reported that nuclear factor  $\kappa$ B (NF $\kappa$ B), which plays pivotal roles in inflammation mediated by various cytokines, is activated by aldosterone and that its activation plays a key role in the

regulation of electrolyte transporters in collecting duct cells [2, 3]. It has also been reported that NF $\kappa$ B is activated in cardiomyocytes [4] and cultured mesangial cells [5] and mediates the pro-inflammatory and fibrotic actions of aldosterone. It has been suggested that aldosterone activates AP-1 as well as NF $\kappa$ B in cultured renal fibroblasts [6] and induces osteopontin expression, which in turn increases expression of collagen types 1, 3, and 5. In contrast to the findings in these studies, aldosterone has been found to prevent NF $\kappa$ B-induced tumor necrosis factor (TNF)- $\alpha$  production in neutrophils [7], suggesting that the relationship between aldosterone and NF $\kappa$ B activation may vary with the tissue and cell type. In the present study we investigated whether the pro-inflammatory actions and fibrotic actions of aldosterone are mediated by NF $\kappa$ B activation in in-vivo experiments, and changes in expression of angiotensin-converting enzyme 2 (ACE2), which is known to act as protective factor in various kidney injuries, were also determined in the aldosterone-infused rats.

## Materials and methods

### Animals

Male Wistar rats (6–7 weeks of age) were used in this study, and all experimental procedures were approved by the Institutional Laboratory Animal Care and Use Committees of Keio University School of Medicine. Each animal was housed in an individual metabolic cage in a room that was lit 12 h a day and maintained at an ambient temperature of 22°C. Animals were allowed to adjust to the metabolic cage for 4–7 days after delivery, and they were given free access to food and tap water until the start of the experiment.

All rats were subjected to left nephrectomy under pentobarbital anesthesia, and an osmotic minipump (Alzet model 2004, Durect Corp., Cupertino, CA, USA) containing either vehicle (3% ethanol in ddH<sub>2</sub>O) or 3.0 mg/ml D-aldosterone (Sigma Chemical Company, St. Louis, MO, USA; this concentration of aldosterone delivers 0.75  $\mu$ g/h), was inserted subcutaneously between the shoulder blades.

After recovery from surgery, the animals were divided into the following groups: a control group (vehicle infusion and 0.9% NaCl drinking water), an aldosterone group (aldosterone infusion and 0.9% NaCl drinking water), a spironolactone group (aldosterone infusion, spironolactone 200 mg/kg BW/day mixed with powdered chow, and 0.9% NaCl drinking water), and an IMD-1041 group (aldosterone infusion, IMD-1041 100 mg/kg BW/day mixed with powdered rat chow, and 0.9% NaCl drinking water). IMD-1041 is a prodrug of IMD-0354 that was developed by the Institute of Medicinal Molecular Design and specifically

inhibits IKK $\beta$  in in-vivo [8] and in-vitro experiments [9]. In the preliminary experiments, this oral dose of IMD-1041 was confirmed to keep its blood concentration level enough for the inhibition of IKK $\beta$  in the in-vivo condition.

Blood pressure was measured by tail-cuff plethysmography, and urine samples were collected every week for 3 weeks after the start of aldosterone infusion. All rats were killed under pentobarbital anesthesia 4–7 days after the final measurements. Blood samples were taken from the aorta after pentobarbital anesthesia for the measurement of serum creatinine levels. The remaining kidney was then perfused with cold saline and removed. Small pieces of cortex were immediately placed in liquid nitrogen for subsequent extraction of protein and total RNA. Samples for histological and immunofluorescence studies were processed according to the methods described in our previous reports [10, 11].

### Histological analysis

Coronal sections of the remaining kidney were immersion-fixed in 10% neutral-buffered formalin and embedded in paraffin. Sections were stained with Masson's trichrome, and then examined by light microscopy. Histological parameters (glomerular injury, arterial injury, and tubulointerstitial changes) were semiquantitatively graded in a blinded manner by two investigators and the mean score was calculated according to a previously described method [12] with minor modification. The severity of each histological parameter was scored as follows: 1, normal; 2, minimal to 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.

### Real-time PCR quantification of mRNA

Frozen tissue samples were immersed in RNAlater-ICE (Ambion, Austin, TX, USA) at  $-80^{\circ}\text{C}$  for 3 days. A 10-mg tissue sample was homogenized in 600  $\mu$ l of Buffer RLT with a Mixer Mill MM 200 (Retsch, Haan, Germany). Total RNA was purified by using an RNeasy Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The RNA concentration was determined by spectrometry with a NanoDrop 1000 spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). First-strand cDNA was generated from 1  $\mu$ g of total RNA by using random hexamer and a SuperScript III First-Strand Synthesis Kit (Invitrogen, Carlsbad, CA, USA). Quantitative real-time PCR assays were performed by Taqman Gene Expression assays using Taqman universal PCR master mix (Applied Biosystems, Foster City, CA, USA). A 10-ng sample of total RNA from each sample (0.1 ng for 18 s rRNA) was used in a 25- $\mu$ l reaction mixture. PCR was

started by an initial 10-min incubation at 95°C for enzyme activation followed by 45 cycles of two-step thermal cycling, 95°C for 15 s and 60°C for 1 min. PCR reaction was monitored by ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Duplicate reactions were performed for each sample. Gene expression was calculated relative to 18S rRNA. The following probes were purchased from Applied Biosystems and used for PCR reaction: PAI-1: Rn00561717\_m1; fibronectin: Rn00569575\_m1; collagen type I: Rn00801649\_g1; osteopontin: Rn01449972\_m1; ACE2: Rn01416293\_m1, 18S, Hs99999901\_s1.

#### Western blot and immunohistochemical analyses

Protein samples were obtained from kidney cortex. An equal amount of protein in each lane was subjected to 10% SDS-polyacrylamide gel-electrophoresis, and the proteins were electrotransferred to a PVDF membrane (BioRad, Hercules, CA, USA). The membrane was blocked with Blocking One (Nacalai Tesque, Kyoto, Japan) and then incubated for 1 h at room temperature with anti-p65 antibody (ab7970; Abcam, Cambridge, MA, USA), anti-Sp1 antibody (sc-59X; Santa Cruz Biotechnology, Santa Cruz, CA, USA), or anti-ACE2 antibody (ab15347; Abcam). Immunodetection was performed using the ECL blotting detection system (GE Healthcare, Buckinghamshire, UK) and ImageMaster VDS-CL (GE Healthcare).

Thin sections (~6 µm) were prepared with a cryotome and used for the immunofluorescence studies. Tissue samples were treated for 15 min at 100°C with Dako REAL Target Retrieval Solution (Dako Co., Glostrup, Denmark) before incubation with the antibodies. Rabbit anti-ACE2 antibody (ab15347; Abcam) was used as the first antibody, and Alexa488-labeled donkey anti-rabbit IgG (A21206; Molecular Probes, Eugene, OR, USA) was used as the second antibody. In the double-staining studies, thin sections were incubated with anti-ACE2 antibody and goat anti-megalin antibody (sc-16478; Santa Cruz Biotechnology) as the first antibodies. Alexa488-labeled donkey anti-goat IgG antibody and Alexa594-labeled donkey anti-rabbit antibody (A11055 and A21207, respectively; Molecular Probes) were used as the second antibodies. In both studies, after incubation with the first antibody at 4°C overnight and four washes with PBS, the tissue sections were stained for 2 h in PBS containing the second antibody. The sections were then washed in PBS (4 times), mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA), and examined by epifluorescence microscopy.

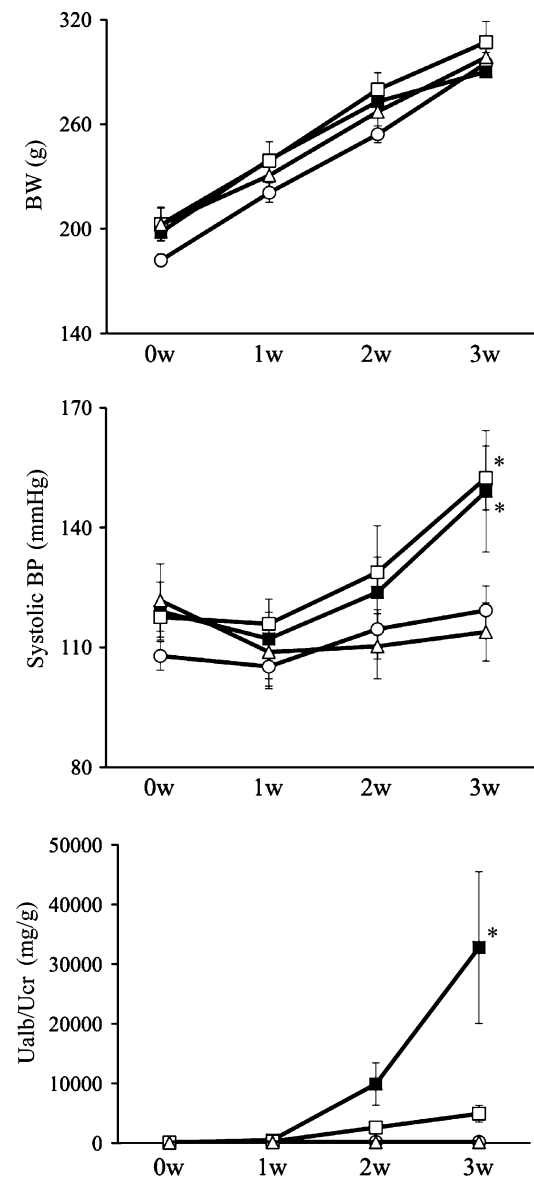
#### Statistics

All data are expressed as mean ± SEM. Multiple parametric comparisons were evaluated by one-way analysis of

variance followed by Tukey–Kramer's test. *P* values less than 0.05 were considered statistically significant.

#### Results

There were no significant differences between the changes in body weight in the four groups (Fig. 1). Systolic blood pressure increased significantly in the aldosterone and IMD-1041 groups in comparison with the control and spironolactone groups. Urinary albumin excretion also



**Fig. 1** Changes in body weight, systolic blood pressure, and urinary albumin excretion in the four groups. \**P* < 0.05 compared with the control group. Data are presented as mean ± SE. Open circles control group, open triangles spironolactone group, open squares IMD10-41 group, closed squares aldosterone group

increased significantly in the aldosterone group in comparison with the control, spironolactone, and IMD-1041 groups, and urinary albumin excretion in the IMD-1041 group was higher than in the control and spironolactone groups, but the differences between the three groups were not statistically significant.

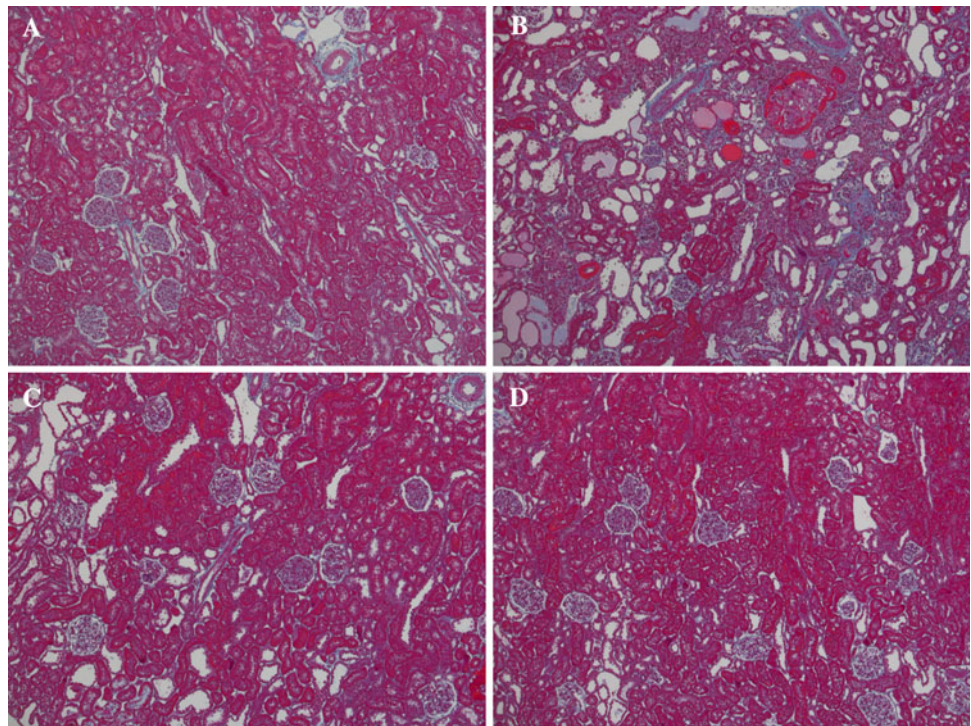
Histological examination of the kidney cortex after Masson's trichrome staining showed clear interstitial fibrosis and glomerular sclerosis in the aldosterone group (Fig. 2b) when compared with the control (Fig. 2a) and the spironolactone groups (Fig. 2d). The inhibitor of NF $\kappa$ B activation, IMD-1041, was found to have mitigated these changes in the kidney (Fig. 2c) when compared with the aldosterone group (Fig. 2b). Semiquantitative analysis revealed significant improvement of histological scores by IMD-1041 and spironolactone (Table 1). Consistent with

histological scores, the increase in serum creatinine levels by aldosterone was prevented by IMD-1041 and spironolactone treatments (Table 1).

Analysis by real-time PCR revealed a significant increase in mRNA expression of osteopontin, fibronectin, PAI-1, and collagen type I in the aldosterone group by comparison with the control group (Fig. 3) and these changes in mRNA expression were completely prevented by spironolactone treatment. These aldosterone-induced changes in mRNA expression were also significantly lessened by IMD-1041, although the differences in mRNA expression from the aldosterone group were smaller in the IMD-1041 group than those in the spironolactone group.

Since the genes that encode them are known to be regulated by NF $\kappa$ B, we attempted to demonstrate activation of NF $\kappa$ B by Western blotting of the p65 in the nuclear

**Fig. 2** Representative photomicrographs of Masson's trichrome-stained kidney cortex from the four groups. **a** Control group, **b** aldosterone group, **c** IMD-1041 group, **d** spironolactone group. Aldosterone and high salt intake induced severe glomerulosclerosis and tubulo-interstitial changes in the kidney, and IMD-1041 markedly mitigated those changes



**Table 1** Serum creatinine concentration and kidney histological scores in four groups

Groups	Control ( $n = 4$ )	Aldosterone ( $n = 10$ )	IMD-1041 ( $n = 11$ )	Spironolactone ( $n = 10$ )
Serum creatinine (mg/dl)	$0.28 \pm 0.02$	$0.45 \pm 0.03^*$	$0.36 \pm 0.05$	$0.31 \pm 0.01^{\S}$
Glomerular injury	$1.07 \pm 0.03$	$2.48 \pm 0.24^{**}$	$1.78 \pm 0.12^{\S}$	$1.65 \pm 0.11^{\S}$
Arterial injury	$1.09 \pm 0.06$	$3.23 \pm 0.32^{**}$	$1.37 \pm 0.27^{\S}$	$1.13 \pm 0.14^{\S\S}$
Tubulo-interstitial injury	$1.01 \pm 0.01$	$2.54 \pm 0.32^{**}$	$1.73 \pm 0.17^{\S}$	$1.60 \pm 0.12^{\S}$

Data are presented as mean  $\pm$  SE

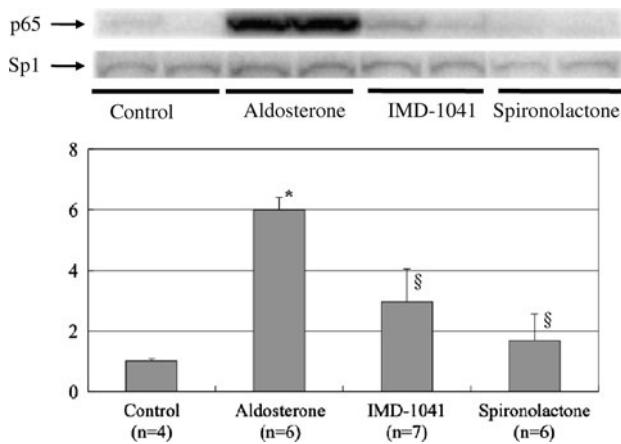
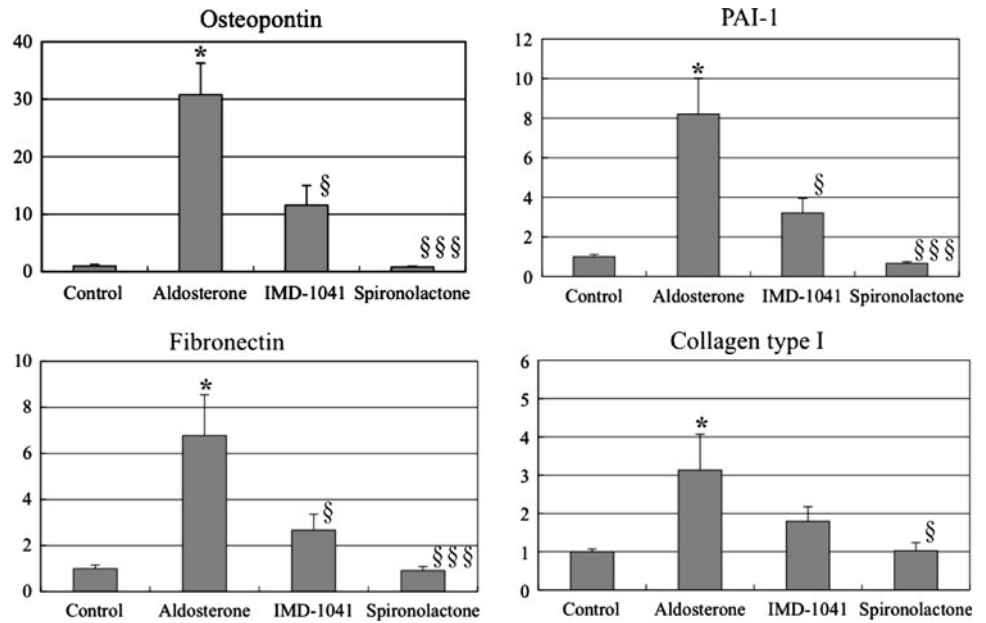
\*  $P < 0.05$  compared with control group

\*\*  $P < 0.01$  compared with control group

$\S$   $P < 0.05$  compared with aldosterone group

$\S\S$   $P < 0.01$  compared with aldosterone group

**Fig. 3** Quantified RT-PCR data in the four groups. \* $P < 0.05$  compared to the control group. § $P < 0.05$  compared with aldosterone group. §§ $P < 0.001$  compared with aldosterone group. The quantified RT-PCR data in the spironolactone ( $n = 10$ ), aldosterone ( $n = 10$ ), and IMD-1041 ( $n = 11$ ) groups were standardized to the data in the control group ( $n = 4$ ), which were set equal to 1. Data are presented as mean  $\pm$  SE



**Fig. 4** Representative Western blots of p65 in the nuclear fraction of the kidney in the four groups are shown in the upper panel. Densitometric analysis data are shown in the lower panel. The quantified band density data in the spironolactone, aldosterone, and IMD-1041 groups were normalized against the value in the control group, set equal to 1. \* $P < 0.05$  compared to the control group. § $P < 0.05$  compared with aldosterone group. Data are presented as mean  $\pm$  SE

fraction. As shown in Fig. 4, the band density of p65 was significantly increased in the aldosterone group in comparison with the control, spironolactone, and IMD1041 groups.

We have previously shown tubulo-interstitial changes and a decrease in ACE2 as a result of protein overload via NF $\kappa$ B activation in the kidney cortex of rats [11], and ACE2 is known to play a protective role against various kidney injuries. To determine whether ACE2 is involved in aldosterone-induced tissue injury, we investigated changes in ACE2 expression in response to aldosterone. The results of the immunofluorescence studies in Fig. 5 revealed the

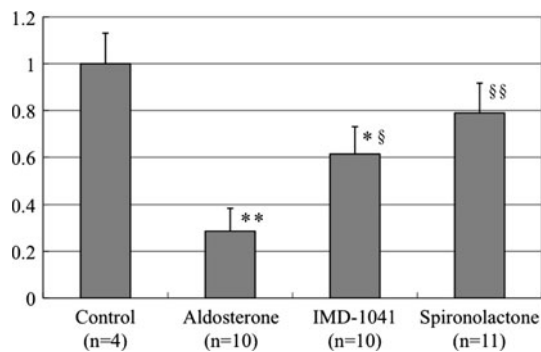
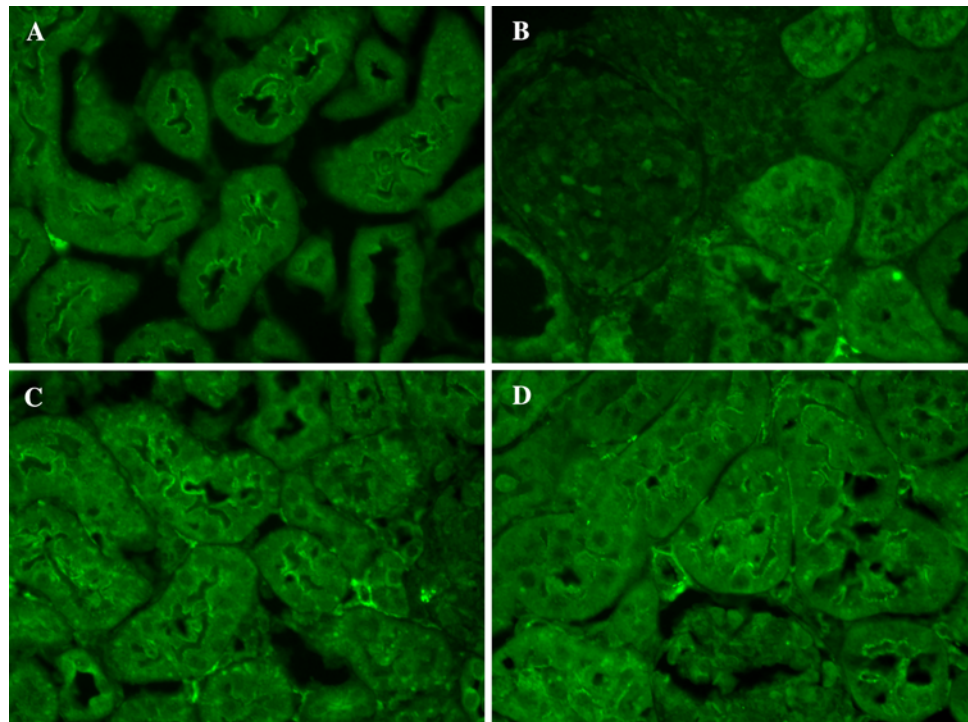
presence of ACE2 on the brush-border membrane of the proximal convoluted tubules (PCT) in the control group and that ACE2 staining was markedly decreased by aldosterone. Inhibition of NF $\kappa$ B activation by IMD-1041 mitigated the decrease in ACE2 staining in the PCT.

The real-time PCR data in Fig. 6 showed a significant decrease in mRNA expression of ACE2 in the aldosterone group in comparison with the control and spironolactone groups. IMD-1041 partially prevented the decrease in ACE2 mRNA expression. Although this effect of IMD-1041 on ACE2 mRNA expression was still significant compared with the aldosterone group, ACE2 mRNA expression in the IMD-1041 group was significantly lower than in the control group. In addition, Western blotting studies showed that ACE2 protein expression was decreased by aldosterone infusion and that spironolactone and IMD-1041 prevented this change (Fig. 7). To determine whether the brush-border membrane itself was injured by aldosterone, we performed double staining of the kidney with anti-ACE2 and anti-megalin antibodies. As shown in Fig. 8, ACE2 staining was faint in the aldosterone group, whereas megalin staining was unchanged, indicating that the decrease in ACE2 expression was not mainly attributable to destruction of the brush-border membrane of the PCT. On the other hand, no ACE2 staining was detected in the mesangial cells of any of the four groups (data not shown).

### Discussion

In this study we investigated whether tissue injury caused by aldosterone is mediated by the NF $\kappa$ B pathway.

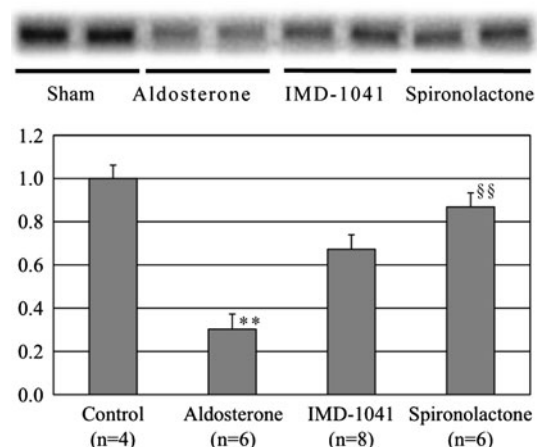
**Fig. 5** Representative photomicrographs of kidney cortex from the four groups stained for ACE2. **a** Control group, **b** aldosterone group, **c** IMD-1041 group, **d** spironolactone group. ACE2 staining is seen mainly on the brush-border membrane and was less intense in the aldosterone group



**Fig. 6** Quantified ACE2 RT-PCR data in the four groups. \* $P < 0.05$  compared with the control group, \*\* $P < 0.01$  compared with the control group. § $P < 0.05$  compared with aldosterone group. §§ $P < 0.01$  compared with aldosterone group. There was no significant difference in ACE2 mRNA expression between the spironolactone and IMD-1041 groups

Following aldosterone infusion and high salt intake, the amount of p65 protein which moves from the cytosolic fraction to the nucleus by NF $\kappa$ B activation was clearly increased in the nuclear fraction of the kidney. The increase in p65 protein in the nucleus was prevented by spironolactone and IMD-1041, but IMD-1041 had no effect on the blood pressure increase induced by aldosterone. The results also clearly showed that systemic NF $\kappa$ B inhibition prevented kidney injury by aldosterone, and that ACE2 expression is down-regulated by the NF $\kappa$ B pathway in this kidney injury model.

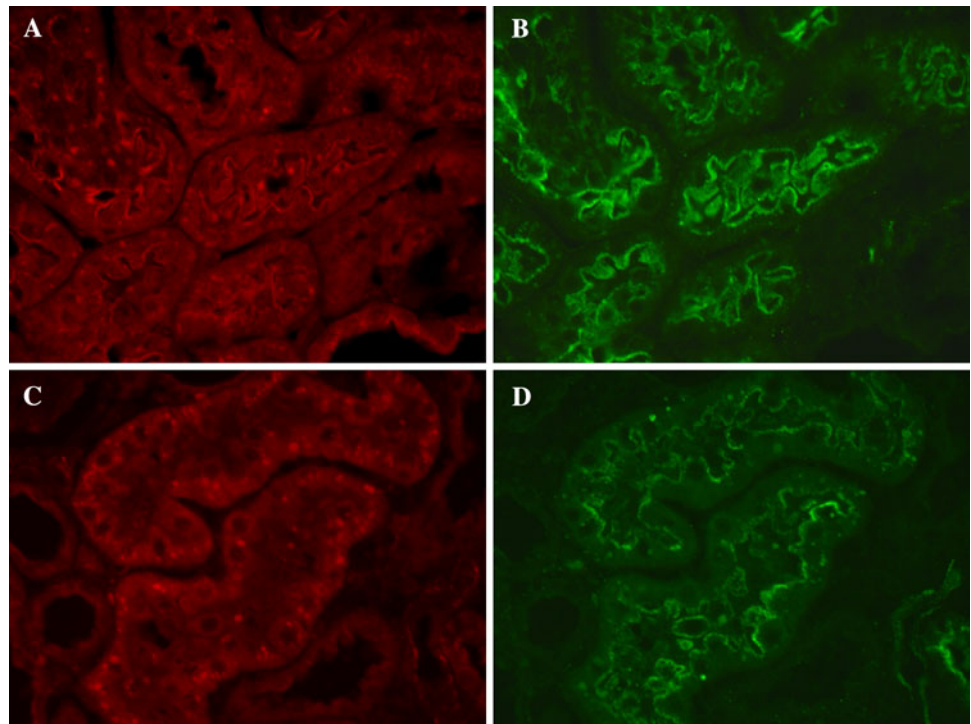
NF $\kappa$ B is known to play a pivotal role as a signal transduction system of various cytokines [13]. In diabetic



**Fig. 7** Representative Western blots of ACE2 in the cytosolic fraction of the kidney in the four groups are shown in the upper panel. Densitometric analysis data are shown in the lower panel. The quantified band density data in the spironolactone, aldosterone, and IMD-1041 groups have been normalized to the data in the control group, set equal to 1. Data are presented as mean  $\pm$  SE. \*\* $P < 0.01$  compared with the control group. §§ $P < 0.01$  compared with aldosterone group

and various renal injuries, it has been reported that NF $\kappa$ B activation was observed not only in experimental animals [14, 15] but also in patients with kidney diseases [16]. In addition, our previous study [10] clearly showed that albumin-overload-induced tissue injury is mediated by NF $\kappa$ B activation. The results of studies regarding the relationship between aldosterone and NF $\kappa$ B activation

**Fig. 8** Representative photomicrographs of kidney cortex from the control and aldosterone groups stained for ACE2 and megalin. **a, c** ACE2, **b, d** megalin, **a, b** control group, **c, d** aldosterone group. Megalin staining is seen on the brush-border membrane in both groups, whereas ACE2 staining is less intense in the aldosterone group



have shown that the relationship between them depends on the cell type. In cultured renal fibroblasts [6] and freshly isolated cortical collecting duct (CCD) cells [2, 3], aldosterone was shown to activate the NF $\kappa$ B pathway, whereas it was found to directly antagonize the action of interleukin (IL)-8 and granulocyte colony stimulating factor (GCSF)-induced NF $\kappa$ B activation in neutrophils [7], and also induced NF $\kappa$ B activation in cultured mesangial cells [5]. Consistent with the results of these in-vitro experiments, the results of the present study showed that aldosterone activates NF $\kappa$ B and that systemic inhibition of NF $\kappa$ B by a specific inhibitor, IMD-1041, mitigated the kidney injury and the increase in mRNA expression of pro-inflammatory and fibrotic cytokines under in-vivo conditions, although the effects of IMD-1041 were partial in these changes of mRNA expression, probably due to at least two mechanisms. The effects of aldosterone are known to be mediated by the AP-1 pathway as well as by NF $\kappa$ B activation [6], and presumably the AP-1 pathway was still active and capable of inducing tissue injury in our experiment. The other possibility for the limited effects of IMD-1041 is that hypertension remained unchanged. The blood pressure levels of the aldosterone-infused rats were not significantly changed by IMD-1041, and high blood pressure could induce kidney injury and changes in mRNA expression of pro-inflammatory and fibrotic cytokines. Despite this limitation, the NF $\kappa$ B inhibitor IMD-1041 may be a good candidate drug for the treatment of diabetic nephropathy and various kidney diseases.

The results of the present study confirmed that pro-inflammatory and fibrotic cytokines and proteins, osteopontin, fibronectin, PAI-1, and collagen type I, are up-regulated by aldosterone in rats, the same as reported previously in aldosterone-infused mice [17], and the data in our study showed that these effects of aldosterone were exerted via NF $\kappa$ B activation. The results also showed that ACE2 is down-regulated by aldosterone via NF $\kappa$ B activation, and the decrease in ACE2 expression was confirmed by real-time PCR and Western blotting. In the immunofluorescence studies, ACE2 staining was mainly seen on the brush-border membranes of the PCT. Colocalization of ACE2 and megalin, a PCT marker protein, confirmed this localization of ACE2. The previous studies consistently demonstrated that ACE2 staining in the rat kidney is in the PCT, whereas the results for glomerular staining with anti-ACE2 antibody have varied [18–22]. No glomerular staining of ACE2 was seen in the present study, and the staining was mainly seen on the brush-border membranes. The reason for the differences in the results for glomerular staining was not investigated in the present study, but the differences in antibodies used in the previous and present studies may be responsible for the discrepancies.

The results of the present study showed that aldosterone down-regulates ACE2 via the NF $\kappa$ B pathway, and the decrease in ACE2 did not appear to have been caused by destruction of the brush-border membrane, because megalin staining, a marker of the brush-border membrane, was

unchanged in the aldosterone group. ACE2 has recently been cloned, and its physiological and pathophysiological roles have been characterized in the lung, kidney, and cardiovascular system [23]. In addition to its role as receptor protein for the SARS virus, ACE2 acts as a protective factor against tissue injury by angiotensin II. Indeed, changes in ACE2 expression and activity have been reported in various kidney injury models. ACE2 expression in the tubules of rats with streptozotocin-induced diabetes has been found to be decreased [21], and kidney biopsy specimens from diabetic patients have shown decreased expression of tubular and glomerular ACE2 [24]. Spontaneously hypertensive rats also show decreased tubular ACE2 expression when they develop hypertension, whereas glomerular expression of ACE2 increased after birth and after the development of hypertension [19]. Our previous study revealed that protein overload induced a decrease in ACE2 expression via NF $\kappa$ B activation [11]. ACE2 expression in cultured neonatal rat cardiomyocytes was significantly decreased by aldosterone, and the specific mineralocorticoid receptor inhibitor eplerenone completely abolished this effect of aldosterone [25], consistent with the results of the present study in the kidney. Furthermore, the results of the present study clearly showed that the decrease in ACE2 protein expression by aldosterone was mitigated by NF $\kappa$ B inhibition. Combining our previous results [11] and the present data, it was suggested that ACE2 expression was down-regulated by NF $\kappa$ B activation. It is possible, however, that the down-regulation of ACE2 by NF $\kappa$ B in this study was induced by the indirect effect of systemic inhibition of NF $\kappa$ B, since IMD-1041 was orally administered. In addition, it was not determined which cells in the kidney were involved in the effects of IMD-1041, for the same reason. Previous reports on tissue injury by aldosterone have clearly shown that inflammation plays important roles in the remodeling of the heart, vasculature, and kidney [26, 27]. Furthermore, emerging evidence suggests that aldosterone receptors are present in various types of cells, including endothelial cells, macrophages, vascular smooth cells, cardiac cells, and renal tubular and glomerular cells, and that activation of the aldosterone receptor on these cells could induce inflammation in the organs [26, 27]. Therefore, it is possible that NF $\kappa$ B was activated by aldosterone and induced inflammation not only in tubular and mesangial cells but also in various intrarenal and extrarenal cells, such as macrophages and endothelial cells, and that the beneficial effects of IMD-1041 treatment, amelioration of kidney injury and the increase in ACE2, were exerted by inhibition of NF $\kappa$ B activity in all of these cell types. On the other hand, in this present study, we did not examine the entire intrarenal renin–angiotensin system, which includes local renin production, ACE activity, and angiotensin metabolism. The

determination of the changes in the intrarenal renin–angiotensin system should shed light on the exact role of ACE2 in aldosterone-induced kidney injury and should be performed in future studies.

In conclusion, the results of this study showed that aldosterone activates NF $\kappa$ B in the kidney and that ACE2 is down-regulated by aldosterone. The effects of aldosterone on pro-inflammatory and fibrotic proteins were abolished by IMD-1041, suggesting that NF $\kappa$ B inhibitors may be novel therapeutic agents for various kidney diseases.

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**Conflict of interest** Kaori Harada, Toshifumi Wakamatsu, Hiroshi Fukasawa, and Susumu Muto are employees of the Institute of Medical Molecular Design Inc. Akiko Itai owns stocks in the Institute of Medical Molecular Design Inc.

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