Original Article

Aldosterone-and-Salt–Induced Cardiac Fibrosis Is Independent from Angiotensin II Type 1a Receptor Signaling in Mice

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Aldosterone infusion with high salt treatment induces cardiac fibrosis in rats. Aldosterone enhanced angiotensin II (Ang II) has been shown to induce proliferation and increase the expression of Ang II receptor mRNA and Ang II binding *in vitro*. To investigate the role of Ang II type 1a receptor (AT1aR) in aldosterone-and-salt (Ald-NaCl)–induced cardiac fibrosis, we subcutaneously infused aldosterone (0.15 μg/h) and 1% NaCl (Ald-NaCl) into AT1aR knockout mice (AT1aR-KO) or wild type mice (Wt). To examine the role of NaCl on cardiac fibrosis, we gave some of the aldosterone-treated AT1aR-KO tap water (Ald-H_2O). Ald-NaCl treatment increased systolic blood pressure and induced cardiac hypertrophy in both strains, whereas there were no such changes in the mice without aldosterone. Severe cardiac fibrosis was seen in Ald-NaCl–treated AT1aR-KO and not in Ald-NaCl–treated Wt. In contrast, Ald-NaCl–treated Wt with co-administration of an active metabolite of olmesartan, the AT1aR antagonist (10 mg/kg/day) did not show cardiac fibrosis. Na^+/H^+ exchanger, and Na^+-K^+ ATPase α2 subunit mRNA were decreased in AT1aR-KO. Na^+/Ca^{2+} exchanger mRNA was lower in AT1aR-KO than Wt and was decreased by Ald-NaCl in both strains. Phosphorylation of epidermal growth factor receptor and extracellular signal–regulated kinase was increased by Ald-NaCl treatment in AT1aR-KO. Connective tissue growth factor (CTGF) and osteopontin mRNA were increased and accumulation of CTGF proteins was seen in the hearts of Ald-NaCl–treated AT1aR-KO. Ald-H_2O–treated AT1aR-KO did not show any cardiac fibrosis. These results suggest that Ald-NaCl–induced cardiac fibrosis required both aldosterone and salt. Because cardiac fibrosis was exaggerated in Ald-NaCl–treated AT1aR-KO but was not seen in Wt treated with Ald-NaCl and olmesartan, AT1aR may not play a primary role in progression of cardiac fibrosis by Ald-NaCl, and gene disruption of AT1aR may have some implications in this model. (*Hypertens Res* 2007; 30: 979–989)

***Key Words*: aldosterone, angiotensin II, knockout mouse, cardiac fibrosis, sodium**

Introduction

The renin angiotensin aldosterone system (RAAS) plays a key role in cardiovascular control through its effects on vascular tone and fluid volume balance. Although aldosterone is the end product of RAAS, relatively little attention has been given to its physiological role compared with that of angiotensin II. A number of studies have demonstrated that mineralocorticoid receptor (MR) exists not only in the adrenal cortex but also in extra-adrenal tissues such as the myocardium and vascular smooth muscle cells (VSMC) (*1–3*).

In 1992, Brilla and Weber reported that cardiac hypertrophy associated with interstitial and perivascular fibrosis occurred following administration of exogenous aldosterone (*4*). Since then, a number of studies have demonstrated the

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Table 1. Primer Sequences

	Forward	Reverse
Angiotensin II type 1a receptor	GGACACTGCCATGCCCATAAC	TGAGTGCGACTTGGCCTTTG
Angiotensin II type 1b receptor	AGCGCCAGCAGCACTGTAGA	ATTGTGCCTGCCAGCCTTG
Angiotensin II type 2 receptor	GTGCATGCGGGAGCTGAGTA	ATTGGTGCCAGTTGCGTTGA
Mineralocorticoid receptor	CGGTCCTAGAGTACATTCCA	CCATAGTGACACCCAGAAGC
Na^+/H^+ exchanger	TTACACTTGGCTTATTTGCACCTCA	CAGGTTGGGCCACATCAGAA
Na^+/Ca^{2+} exchanger	AAGATTCCGTGACTGCCGTTG	ACGCATCTGCATACTGGTCCTG
Na^+/HCO_3^- exchanger	CATCGACAGTTTGAAGATGGAGACA	TAAGCACAAGGGTTCCGGTGA
Voltage-gated Na channel	AATGTCTCAGCCTTACGCACCTTC	AGGGCTCCCACGATTGTCTTC
Na^+-K^+ ATPase α1 subunit	CCAAGCAAGCTGCTGACATGA	TACTGGTTAGGGTGTAAGCGATGGA
Na^+-K^+ ATPase α2 subunit	AACCAGATCCATGAGGCTGACAC	TTGAAGACAGCACGATTGCAGAG
Connective tissue growth factor	ACCCGAGTTACCAATGACAATACC	CCGCAGAACTTAGCCCTGTATG
Osteopontin	TACGACCATGAGATTGGCAGTGA	TATAGGATCTGGGTGCAGGCTGTAA
GAPDH	AAATGGTGAAGGTCGGTGTG	TGAAGGGGTCGTTGATGG

association of cardiac fibrosis with aldosterone and salt excess (*5*, *6*). Several large-scale clinical studies demonstrated the clinical benefits of MR antagonists; the Cooperative North Scandinavian Enalapril Survival Study (CONSENSUS) showed a relationship between the plasma aldosterone concentration and mortality (*7*), and in the Randomized Aldactone Evaluation Study (RALES) (*8*) and the Eplerenone Postacute Myocardial Infarction Heart Failure Efficacy and Survival Study (EPHESUS) (*9*), aldosterone inhibition reduced the mortality in patients with congestive heart failure.

It has been established that angiotensin II stimulates the secretion of aldosterone from the zona glomerulosa of the adrenal gland; however, several recent *in vitro* and *in vivo* studies reported that cardiac aldosterone also increased the expression of the components of RAAS (*10–13*). Incubation of the rat VSMC with aldosterone increased angiotensin II receptor binding and angiotensin II type 1a receptor (AT1aR) mRNA (*12*). In another study using rats, angiotensin II–induced cardiac hypertrophy and fibrosis were found to be partly mediated by aldosterone, because both an MR antagonist and an angiotensin II type 1 receptor (AT1R) antagonist equally inhibited the cardiovascular damage induced by angiotensin II (*13*). These results indicate that aldosterone may enhance the angiotensin II–mediated signaling pathway, which might induce cardiac remodeling.

It is well-known that several intracellular signaling pathways can be stimulated by aldosterone. In the present study we focused on the epidermal growth factor receptor (EGFR)–mediated extracellular signal–regulated kinase (ERK) transactivation, connective tissue growth factor (CTGF) and osteopontin. We previously reported that EGFR-mediated ERK transactivation was increased in angiotensin II–infused rats (*14*) and spontaneously hypertensive rats (*15*), and Nakano *et al*. revealed that this pathway was activated by aldosterone and salt treatment (*16*). In addition, the expression of CTGF and osteopontin has been shown to be increased by aldosterone treatment (*17*, *18*). Furthermore, the cardiac fibrosis induced by aldosterone was ameliorated in osteopontin-deficient mice (*19*).

Based on the findings that aldosterone stimulates angiotensin II receptor expression and the signaling pathway of angiotensin II receptor, we hypothesized that the cardiac fibrosis induced by aldosterone may be attenuated by the blockade of AT1R signaling. Accordingly, in the present study, to investigate the role of AT1R signaling in aldosterone-induced cardiac remodeling, we infused aldosterone exogenously into AT1aR knockout mice (AT1aR-KO) and wild-type mice (Wt).

Methods

Experimental Protocol

The experimental protocol was approved by the Committee on the Ethics of Animal Experimentation of the Faculty of Medicine, Kyushu University.

AT1aR-KO (9 weeks old, $n=19$) were kept on a normal light/dark cycle with standard mouse chow (0.4% NaCl). Aldosterone (0.15 μg/h; Wako Chemicals, Osaka, Japan) or its vehicle as a control (polyethylene glycol: PEG 300; Sigma, St. Louis, USA) was administered to AT1aR-KO ($n=6$ for each group) or control mice (C57BL/6J, 9–11 weeks old, $n=6$ for each group) by an osmotic minipump (model 1002; Alzet, Cupertino, USA) for 4 weeks. The osmotic minipumps were replaced every 2 weeks. These mice were kept on 1% NaCl *via* the drinking water. In addition, some of the AT1aR-KO ($n=7$) given aldosterone were given tap water without NaCl (Ald-H_2O) to investigate the role of NaCl in this model. Furthermore, to investigate the effects of pharmacological blockade of AT1R signaling on aldosterone-and-salt (Ald-NaCl)–induced cardiac fibrosis, Wt administered aldosterone and 1% NaCl were additionally treated with RNH-6270, which is an active metabolite of the AT1R antagonist

olmesartan (10 mg/kg/day). RNH-6270 was provided by Sankyo Pharmaceuticals (Tokyo, Japan) and was dissolved in drinking water with 1% NaCl. Systolic blood pressure (SBP) was measured by the conventional tail cuff system (Muromachi Kikai, Tokyo, Japan). After 4 weeks of treatment, mice were deeply anesthetized by pentobarbital (50 mg/kg, i.p.). Blood samples were taken *via* transcardiac puncture, and the heart was removed and weighed. The serum aldosterone concentration was measured by radioimmunoassay. To evaluate cardiac fibrosis, we visualized Masson-trichrome–stained sections (4 μm) by light microscopy and calculated the ratio of the fibrotic area to the total myocardial area using Scion Image.

Quantitative Real-Time Polymerase Chain Reaction Analysis

Total RNA was isolated from the hearts with an ISOGEN (Nippon Gene, Tokyo, Japan). The mRNA expressions were analyzed by real-time quantitative polymerase chain reaction (PCR) performed with a TaqMan system with 75 ng of total RNA based on real-time detection of accumulated fluorescence (LightCycler; Roche, Basel, Switzerland) using a One-Step SYBR RT-PCR kit (Takara Bio Inc., Otsu, Japan). The expressions of mRNA were evaluated with the GAPDH gene as an endogenous control. Primers were constructed using a Perfect Real-Time Support System (Takara Bio Inc.) or by the Nihon Gene Institute (Sendai, Japan). The primer sequences are shown in Table 1. The amplification was performed with the following time course: 15 min at 42°C and 2 min at 95°C; and 40 cycles of 5 s at 95°C and 20 s at 60°C. Results were expressed as relative to the values for Wt without aldosterone treatment, which were arbitrarily assigned a value of 1.0.

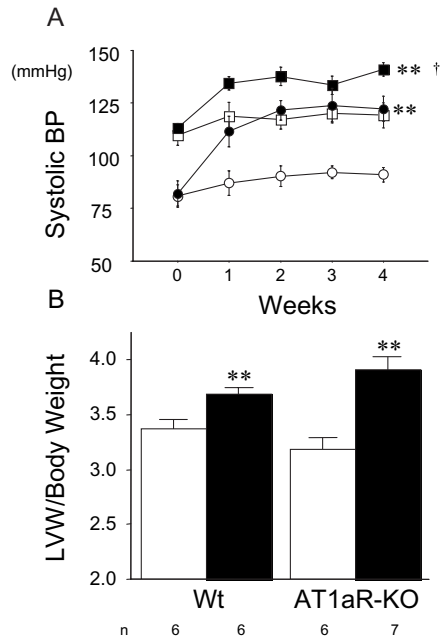

Fig. 1. *A: Time course of changes in systolic blood pressure (SBP) in AT1aR-KO treated with NaCl alone (open circles), AT1aR-KO treated with aldosterone and NaCl (closed circles), Wt treated with NaCl alone (open squares), and Wt treated with aldosterone and NaCl (closed squares). **$p < 0.01$ vs. mice without aldosterone treatment. †$p < 0.05$ vs. AT1aR-KO treated with aldosterone. B: Left ventricular weights/body weights in AT1aR-KO treated with NaCl alone (open bars), AT1aR-KO treated with aldosterone and NaCl (closed bars), Wt treated with NaCl alone (open bars), and Wt treated with aldosterone and NaCl (closed bars). **$p < 0.01$ vs. mice without aldosterone treatment.* Ã*, 1% NaCl;* Â*, 1% NaCl+aldosterone.*

Western Blot Analysis

The left ventricle (LV) was homogenized in ice-cold lysis buffer (25 mmol/L Tris-HCl [pH 7.4], 25 mmol/L NaCl, 10 mmol/L NaF, and 1 mmol/L sodium orthovanadate in Complete Mini Peptidase Inhibitor Cocktail [Roche]), and centrifuged for 10 min at 13,000 × *g* and 4°C. Aliquots of proteins (60 μg) were subjected to sodium dodecyl sulfate–polyacrylamide gel (10%) electrophoresis, and transferred onto nitrocellulose membranes (ECL nitrocellulose; GE Healthcare UK Ltd., Buckinghamshire, UK). Immunoblotting was performed with a One-Step Western Blot Kit (GenScript Corporation, Piscataway, USA) and enhanced chemiluminescence (ECL plus; GE Healthcare UK Ltd.) using anti–phosphor-specific EGFR antibody (1:2,000; Cell Signaling Technology Inc., Danvers, USA), anti-EGFR antibody (1:2,000; Santa Cruz Biotechnology, Santa Cruz, USA), anti–phosphor-specific ERK2 (1:2,000, p-ERK; Santa Cruz Biotechnology), anti-ERK2 antibody (1:5,000; Santa Cruz Biotechnology), anti-CTGF antibody (1:3,000; Abcam Plc, Cambridge, UK) and anti-GAPDH antibody (1:10,000; Ambion, Foster City, USA). The blots were repeated at least twice.

Immunohistochemistry

Hearts from each group of mice were used for the immunohistochemical experiments ($n=3$). The 10% formaldehyde–fixed hearts were embedded in paraffin, cut into 4-μm thick sections, deparaffinized, and rehydrated in graded alcohols. The slides were treated in a microwave for 15 min in 10 mmol/L citrate solution buffer for antigen retrieval, and then incubated with 0.3% H_2O_2 in methyl alcohol for 30 min to suppress endogenous peroxidase activity. A rabbit polyclonal antibody specific to CTGF (1:200; Abcam Plc) was applied at 4°C overnight. After being washed with PBS, the slides were incubated with biotinylated goat anti-rabbit IgG (Histofine SAB-PO (R) Kit; Nichirei, Tokyo, Japan) for 30 min at room temperature. The immunoreactivity was visualized by the streptavidin peroxidase staining method.

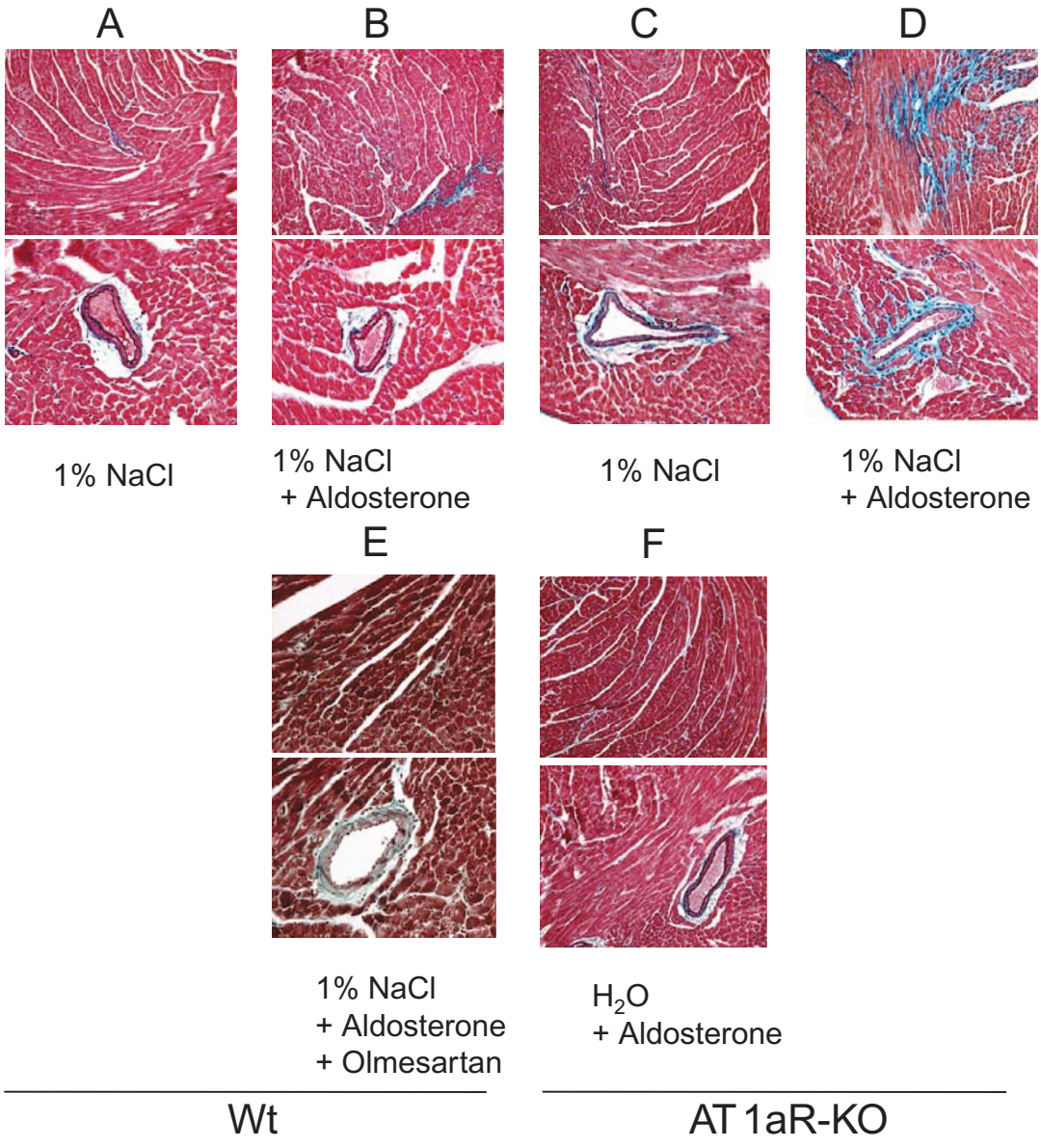

Fig. 2. *Masson trichrome staining of the heart. A: Wt treated with NaCl alone. B: Wt treated with aldosterone and NaCl. C: AT1aR-KO treated with NaCl alone. D: AT1aR-KO treated with aldosterone and NaCl. E: Wt treated with aldosterone, NaCl and RNH-6270. F: AT1aR-KO treated with aldosterone but without NaCl. Magnification, ×200.*

Statistics

Data are expressed as the mean±SEM. Differences between the treatments and species were analyzed by two-way ANOVA followed by Dunnett's test, and other statistical analyses were performed by Student's *t*-test. Values of $p<0.05$ were considered to indicate statistical significance.

Results

Effects of Aldosterone on Blood Pressure and Cardiac Hypertrophy in AT1aR-KO Mice

The baseline SBP of AT1aR-KO ($n=19$) was significantly lower than that of Wt ($n=12$; 81.6±3.8 *vs.* 111.7±2.4 mmHg, $p<0.01$). Treatment with NaCl alone did not change SBP in either AT1aR-KO or Wt, but Ald-NaCl treatment increased SBP in both AT1aR-KO ($p<0.01$) and Wt ($p<0.01$). After

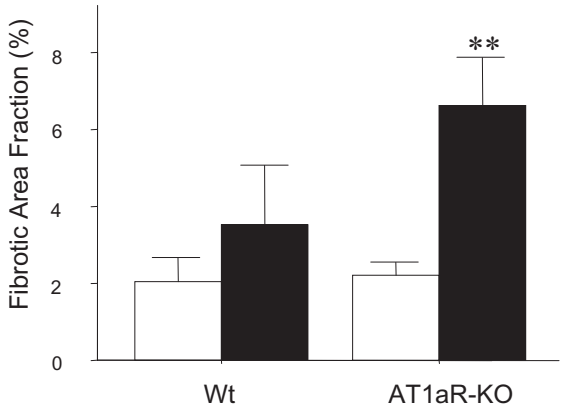

Fig. 3. *Evaluation of the fibrotic area in the heart of AT1aR-KO treated with NaCl alone (open bar), AT1aR-KO treated with aldosterone and NaCl (closed bar), Wt treated with NaCl alone (open bars), and Wt treated with aldosterone and NaCl (closed bars).* ***p*<*0.01 vs. mice treated with NaCl alone.* Ã*, aldosterone (–),* Â*, aldosterone (+).*

Ald-NaCl treatment, the SBP in both strains was significantly higher than that in mice not receiving aldosterone, but the SBP in Ald-NaCl–treated AT1aR-KO was still significantly ($p<0.05$) lower than that in Wt (Fig. 1A). LV weights/body weights (LVW/BW) in Ald-NaCl–treated AT1aR-KO and Wt were significantly higher than in the mice without aldosterone (Fig. 1B) but did not differ between the strains. As a result of aldosterone infusion, plasma aldosterone was increased in AT1aR-KO (722±197 *vs*. 1,805±249 pg/mL) and Wt (285±69 *vs*. 4,170±2,310 pg/mL), and did not differ between strains.

Cardiac Fibrosis in AT1aR-KO Mice

Treatment with NaCl but without aldosterone did not induce any cardiac fibrosis in either strain (Fig. 2A, C). Ald-NaCl–treated Wt showed mild fibrosis in interstitial cardiac tissues but not in the perivascular regions (Fig. 2B). On the other hand, Ald-NaCl–treated AT1aR-KO showed severe fibrosis in both the interstitial and perivascular regions (Fig. 2D). The collagen volume fraction in the heart was significantly increased in AT1aR-KO ($p<0.01$) but not in Wt (Fig. 3).

Effect of Aldosterone and Salt on Wt Treated with AT1R Antagonist

Because genetic disruption of the AT1R induces salt sensitivity (*20*) or blood pressure (BP) reduction in the mice, we investigated whether Ald-NaCl treatment could induce cardiac fibrosis in Wt with pharmacological blockade of AT1R. The SBP (129.0±4.0 mmHg) and LVW/BW (3.79±0.14) in Wt treated with Ald-NaCl and RNH-6270 were significantly increased compared with those of the Wt given NaCl alone. However, neither group of mice showed any cardiac fibrosis (Fig. 2E).

mRNA Expression Profile in the Heart

Because aldosterone has been reported to increase the expression of AT1aR mRNA in the heart (*12*), we examined the change in mRNA expression of the RAAS components in this study (Fig. 4A–D). We did not detect AT1aR mRNA in AT1aR-KO, and the level of AT1aR mRNA in Wt did not change as a result of aldosterone treatment. The expression of angiotensin II type 1b receptor (AT1bR) was decreased in AT1aR-KO and did not change in response to aldosterone treatment. The level of MR mRNA was lower in AT1aR-KO and was decreased by Ald-NaCl treatment. Aldosterone is known to affect the sodium transport through the regulation of the expression of the genes (Fig. 4E–J). We performed real time reverse transcriptional (RT)-PCR to investigate the effect of Ald-NaCl treatment on the expressions of mRNA of sodium-handling–related genes in cardiac tissues. The major Na^+ extrusion pathway in cardiac cells is Na^+-K^+ ATPase. The expression of the α2 subunit of Na^+-K^+ ATPase was decreased in AT1aR-KO (Fig. 4F), but that of the α1 subunit was not changed (Fig. 4E). The major Na^+ influx pathways in cardiac cells are the voltage-gated Na^+ channel, Na^+/H^+ exchanger, and Na^+/HCO_3^- cotransporter. The level of the Na^+/H^+ exchanger was decreased in AT1aR-KO, but the other two molecules were unchanged (Fig. 4G–I). The Na^+/Ca^{2+} exchanger, which has a bidirectional role in Na^+ and Ca^{2+} handling, was decreased in AT1aR-KO and was further decreased by aldosterone treatment (Fig. 4J).

We also investigated the expressions of CTGF and osteopontin in the heart (Fig. 4K, L). Expressions of CTGF and osteopontin mRNA were not different between the strains without treatment of Ald-NaCl. Both mRNAs were increased in Ald-NaCl–treated AT1aR-KO, but not in Ald-NaCl–treated Wt.

Western Blot in the Heart and Immunohistochemistry

The expression of protein was also examined by Western blot. Basal phosphorylation levels of EGFR and ERK were decreased in AT1aR-KO (Fig. 5A–C). The ERK and EGFR were found to be activated in Ald-NaCl–treated AT1aR-KO, but not in Ald-NaCl–treated Wt. CTGF protein levels were higher in Ald-NaCl–treated AT1aR-KO but not in Ald-NaCl–treated Wt compared to the respective strains treated with NaCl but without aldosterone (Fig. 5A, D).

Immunohistochemical staining confirmed the results of the Western blot. CTGF expression was not seen in Wt with or without Ald-NaCl treatment (Fig. 6A, B). In AT1aR-KO, CTGF expression was slightly seen without Ald-NaCl treatment (Fig. 6C). In contrast, an apparent increase in the expression of CTGF was seen in the myocardium of Ald-NaCl–treated AT1aR-KO (Fig. 6D).

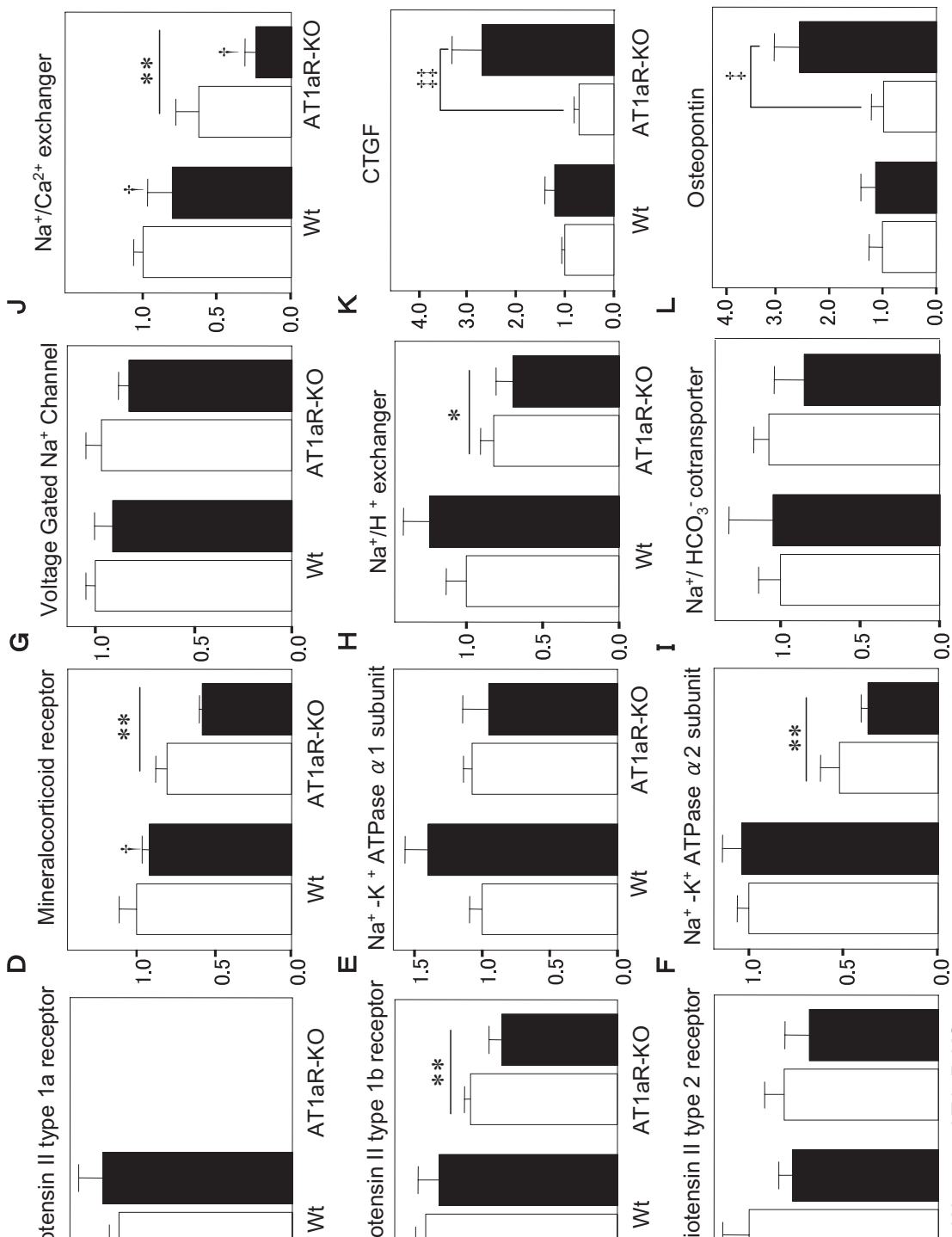

Fig. 4. *Effect of aldosterone and NaCl treatment on mRNA expression in the heart. mRNA expression in the heart analyzed by real-time quantitative RT-PCR. A: angiotensin II type 1a receptor; B: angiotensin II type 1b receptor; C: angiotensin II type 2 receptor; D: mineralocorticoid receptor; E: Na^+-K^+ ATPase α1 subunit; F: Na^+-K^+ ATPase α2 subunit; G: voltage-gated Na^+ channel; H: Na^+/H^+ exchanger; I: Na^+/HCO_3^- cotransporter; J: Na^+/Ca^{2+} exchanger; K: connective tissue growth factor (CTGF); L: osteopontin. The closed bars represent the mice treated with aldosterone and NaCl. The open bars represent the mice treated with NaCl alone. $^*p<0.05$, $^{**}p<0.01$ vs. Wt. $^\dagger p<0.05$ vs. mice with NaCl alone. $^\ddagger p<0.05$, $^{\ddagger\ddagger}p<0.01$.*

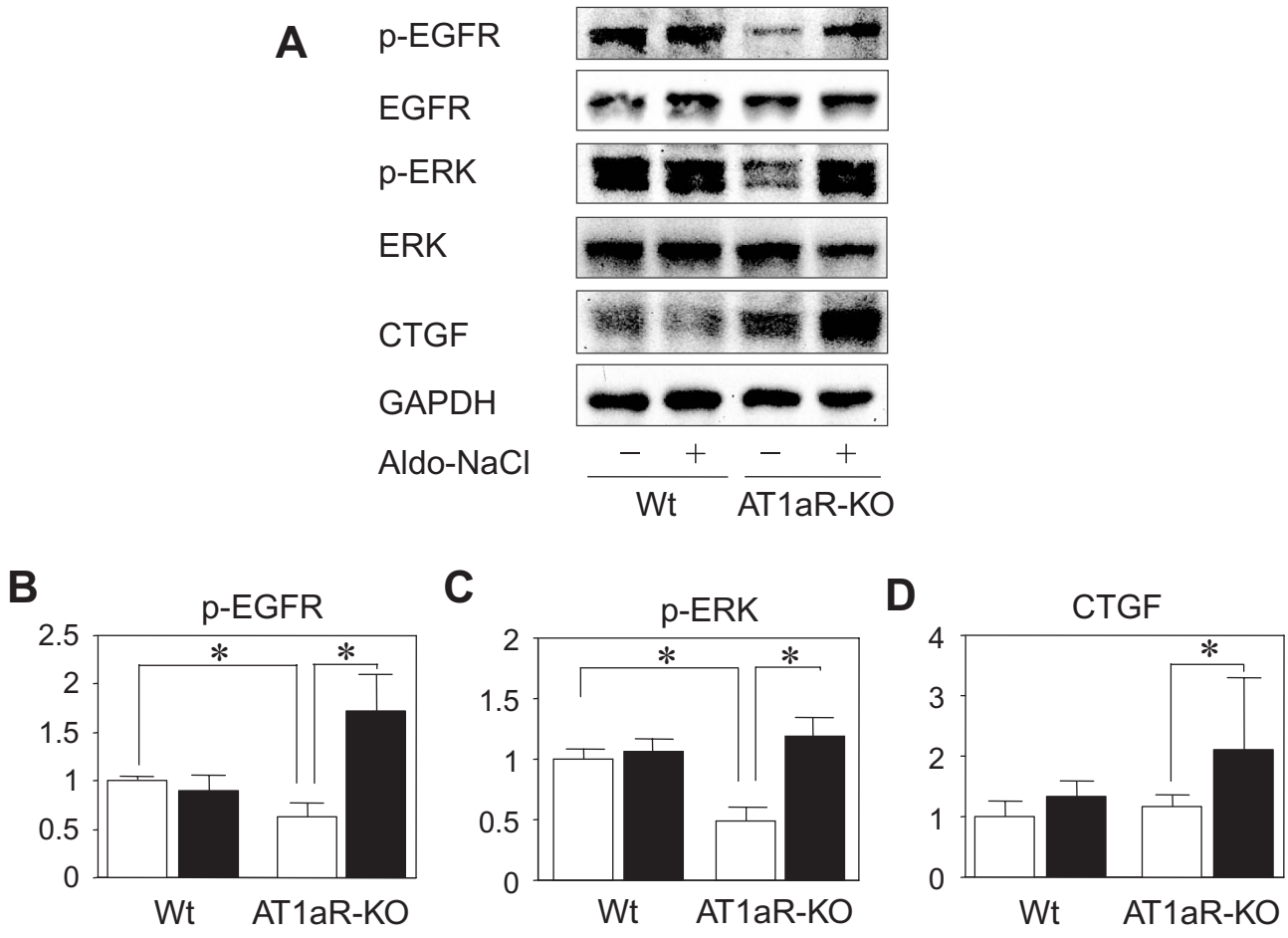

Fig. 5. *Effect of aldosterone and NaCl treatment on protein expression in the heart. A: Representative Western blot. B: The relative density of p-EGFR protein levels to EGFR protein levels. C: The relative density of p-ERK protein levels to ERK-2 protein levels. D: The relative density of CTGF protein levels to GAPDH protein levels. The closed bars represent the mice treated with aldosterone and NaCl. The open bars represent the mice treated with NaCl alone. The protein expression was examined as a fold comparison to Wt with treatment of NaCl alone. *p < 0.05.*

Role of Salt in Aldosterone-Induced Cardiac Fibrosis

As described above, cardiac fibrosis was observed in AT1aR-KO mice receiving both aldosterone and NaCl. To clarify whether NaCl loading is essential in aldosterone-induced cardiac fibrosis, some of the aldosterone-treated AT1aR-KO were given plain tap water. We did not include a group of Wt treated only with aldosterone because we observed severe cardiac fibrosis only in AT1aR-KO (Fig. 2F). Neither the elevation of SBP (96.8±7.7 mmHg) nor the increase in LVW/BW (3.20±0.13) was observed in Ald-H_2O–treated AT1aR-KO, and these mice did not show any cardiac fibrosis.

Discussion

In the present study, we demonstrated that 1) Ald-NaCl treatment increased SBP with cardiac hypertrophy in both Wt and AT1aR-KO; 2) Ald-NaCl treatment caused severe cardiac fibrosis in AT1aR-KO but not in Wt; 3) Ald-H_2O treatment did not increase SBP, and did not induce cardiac hypertrophy or fibrosis; 4) Ald-NaCl treatment did not induce cardiac fibrosis in AT1R-antagonist–treated Wt; and 5) the expression of several Na^+-handling genes was altered by Ald-NaCl treatment and these changes differed between the strains.

Angiotensin II Receptor and Aldosterone

It has been well established that angiotensin II stimulates aldosterone release from the zona glomerulosa of the adrenal gland; however, it is not fully understood whether aldosterone stimulates angiotensin II signaling. Mineralocorticoid-binding sites in VSMC (*3*) and cardiac fibroblasts (*1*) have been identified, and MR-mediated gene transcription was reported to be regulated by both angiotensin II and aldosterone in VSMC (*21*). Angiotensin II–mediated activation of the ERK

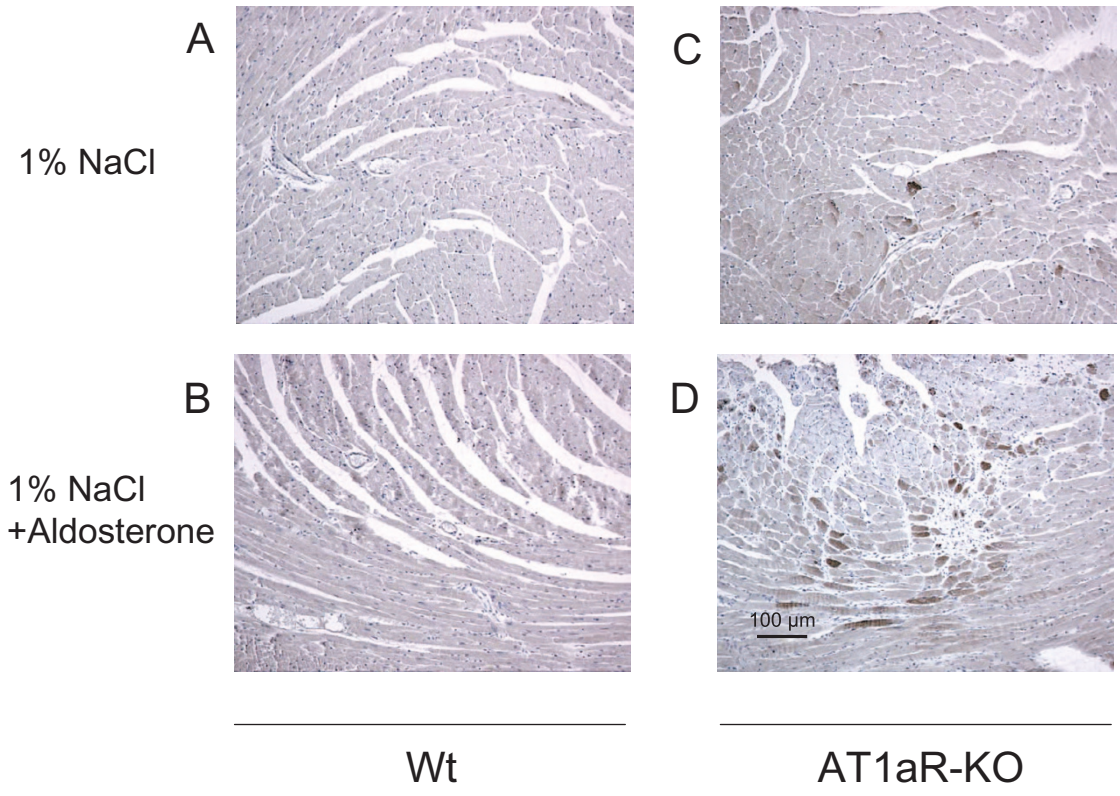

Fig. 6. *Expression of CTGF in the heart as detected by immunohistochemical staining. A: Wt treated with NaCl alone. B: Wt treated with aldosterone and NaCl. C: AT1aR-KO treated with NaCl alone. D: AT1aR-KO treated with aldosterone and NaCl.*

pathway and thymidine incorporation were enhanced by co-incubation of aldosterone with concomitant increases in mRNA and protein expression of AT1aR (*22*). An *in vivo* study demonstrated that aldosterone infusion increased AT1R binding in the rat heart (*23*). Furthermore, both angiotensin converting enzyme inhibitors (ACEIs) and AT1R antagonists have been reported to confer protection against Ald-NaCl–induced cardiac fibrosis (*24*). These results suggested that the angiotensin II signaling pathway plays a pivotal role in aldosterone-mediated cardiac fibrosis.

In the present study, Ald-NaCl–induced cardiac fibrosis appeared to be more severe in AT1aR-KO than in Wt. This was unexpected, but suggests that AT1aR may not be a prerequisite for Ald-NaCl–induced cardiac fibrosis. Elevation of BP also might not be necessary for Ald-NaCl–induced cardiac fibrosis, because in the present study, AT1aR-KO treated with Ald-NaCl showed an SBP comparable to that of Wt treated with NaCl alone, which showed much milder cardiac fibrosis. Katada *et al*. recently reported the importance of aldosterone on cardiac remodeling after myocardial infarction in AT1aR-KO (*25*). In their report, induction of myocardial infarction increased the production of aldosterone in the hearts of AT1aR-KO, and spironolactone prevented the cardiac fibrosis with the reduction of gene expression of collagens and natriuretic peptides (*25*).

Large clinical studies have revealed that treatment with MR antagonists is beneficial in patients with heart failure (*8*, *9*). Our present results indicate that aldosterone itself plays a key role in cardiac fibrosis, and may lend experimental support to the clinical evidence that MR antagonists might be effective in preventing cardiac remodeling in patients with heart failure or myocardial infarction.

In the present study, we demonstrated that Aldo-NaCl could not induce cardiac fibrosis in mice with pharmacological blockade of AT1R. Although it might be suspected that the amount of RNH-6270 was not sufficient to block the AT1R signaling, the amount of olmesartan was the same as used in the study of Fan *et al*. (*17*). The results suggested that the inhibition of AT1R signaling did not worsen the fibrosis in mice, but genetic disruption of AT1R itself is important in Ald-NaCl–induced cardiac fibrosis. We cannot provide a precise explanation for the difference in Ald-NaCl–induced cardiac fibrosis between AT1aR-KO and Wt with pharmacological blockade of AT1R, but we plan to investigate the reasons for this divergence in a future study.

Aldosterone Induces Cardiac Fibrosis in Mice

In 1965, Hall and Hall reported that aldosterone infusion increased BP in Sprague Dawley rats (*26*). They compared

uninephrectomized and intact rats, and revealed that the uninephrectomized rats showed significantly greater BP elevation after aldosterone infusion than rats without nephrectomy. However, they did not examine cardiac fibrosis. Nephrectomy was routinely done in the earlier studies of the aldosterone-infused cardiac fibrosis in deoxycorticosterone acetate hypertensive rats. Yoshida *et al*. reported that aldosterone infusion could induce cardiac hypertrophy and fibrosis in rats without nephrectomy (*27*). In the present study, Ald-NaCl induced cardiac fibrosis in mice without nephrectomy. Taken together, these results suggest that nephrectomy may not be needed to investigate the effect of Ald-NaCl on cardiac fibrosis or remodeling *in vivo*.

Whether aldosterone can induce cardiac fibrosis in mice is still controversial. Ma *et al*. reported that aldosterone failed to induce cardiac fibrosis in Wt (*28*). These authors documented that there might be species differences between mice and rats in terms of the response to Ald-NaCl–induced cardiac fibrosis. There have been several studies on aldosterone-induced cardiac fibrosis in mice (*19*). Previous studies reported that the cardiac fibrosis occurred in Wt after 4 weeks of Ald-NaCl treatment. The amount of aldosterone in the present study corresponds with that in their study, but we did not observe cardiac fibrosis in Wt. It is possible that a species (black Swiss *vs*. C57BL/6J) difference might be involved, because even among rats, sensitivity to Ald-NaCl differs between species (*29*).

mRNA and Protein Expression

At the transcription level, AT1bR and MR mRNA were decreased. The plasma angiotensin II level is increased in this model of mice, and an amount of aldosterone higher than the physiological range was infused in the present study. The results may indicate that the receptors were downregulated as a result of the increase in their agonists. We do not know the physiological meaning of the downregulation of these receptors; however, in the future we plan to treat this mouse model with an MR antagonist.

In the present study, loading of NaCl in addition to aldosterone has a pivotal role in cardiac fibrosis. Aldosterone modulates Na handling by the epithelial Na channel and Na^+-K^+ ATPase in the kidney. In the heart, Na^+-K^+ ATPase is a key target molecule for the cardiac glycoside, and the cardiac glycoside increases intracellular Ca^{2+} by activation of the Na^+/Ca^{2+} exchanger. We observed a decrease in mRNA of the Na^+/H^+ exchanger, Na^+/Ca^{2+} exchanger, and a decrease of the $\alpha 2$ subunit of the Na^+-K^+ ATPase by aldosterone in Wt. Yamamuro *et al*. reported that the incubation of cardiomyocytes under elevated extracellular Na^+ decreased pH, and that an Na^+/H^+ exchanger inhibitor suppressed these change (*30*). The decreased expression of the Na^+/Ca^{2+} exchanger observed in the present study was consistent with that noted in a previous study (*31*).

An aldosterone-induced activation of EGFR-mediated ERK transactivation was previously reported in rats (*16*). Although our findings were not consistent with these previous results because we did not observe the activation of EGFR-mediated ERK transactivation in Ald-NaCl–treated Wt, we did not observe the cardiac fibrosis in Ald-NaCl–treated Wt. The basal levels of EGFR and ERK phosphorylation were decreased in AT1aR-KO and the EGFR-mediated ERK transactivation was increased in Ald-NaCl–treated AT1aR-KO but not in Wt.

CTGF is overexpressed in fibrotic disorders and is reported to be increased by aldosterone or deoxycorticosterone acetate treatment in rats (*17*). Romero *et al*. reported that transforming growth factor β (TGF-β) stimulated CTGF mRNA transcription by increasing intracellular Ca^{2+}, which was modulated by the Na^+/Ca^{2+} exchanger (*32*). Osteopontin is another pro-inflammatory and pro-fibrotic cytokine and its expression was shown to be increased in atherosclerotic vascular lesions (*33*). Aldosterone increased osteopontin mRNA in rat endothelial cells (*18*), and mice lacking osteopontin showed a reduction in fibrosis after aldosterone infusion (*19*).

Salt Is Indispensable for Aldosterone-Induced Cardiac Fibrosis

In the present study, administration of Ald-H_2O failed to induce cardiac fibrosis in AT1aR-KO mice, which suggests that NaCl is indispensable for aldosterone-induced cardiac fibrosis in AT1aR-KO. Our results are consistent with those of previous studies which showed that aldosterone treatment without salt did not induce any cardiac fibrosis in rats (*4*). In the present study, the drinking volume of tap water was greater in AT1aR-KO than in Wt, and this difference became much larger under the NaCl-loading condition. In fact, the total amount of NaCl intake in AT1aR-KO was almost three times as much as that of Wt (data not shown). It was previously reported that AT1aR-KO are salt-sensitive (*20*). Because AT1aR-KO have a reduced ability to concentrate urine (*34*), they might drink more fluid to drain excess NaCl. We consider that exaggerated NaCl load may play a causal role in cardiac fibrosis in AT1aR-KO. Consistent with this idea, a high salt treatment *in vitro* induced cellular proliferation in VSMC (*30*). Very recently, an AT1R antagonist worsened the renal injury score in Dahl salt-sensitive rats under a high salt diet, even though their SBP was reduced (*35*). Based on the findings, we speculate that the absence of AT1R signaling may not play a major role in the cardiac fibrosis induced by aldosterone and salt overload.

Perspective

In the present study, we demonstrated that Ald-NaCl increased cardiac fibrosis in AT1a-KO with some alterations in the Na-handling gene expression, including decreased expression of the Na^+/Ca^{2+} exchanger. Recently, it was reported that an Na^+/Ca^{2+} exchanger inhibitor lowered the BP in salt-dependent hypertension (*36*). This finding indicates

that the salt-dependent hypertension may be at least partly involved in the Ca^{2+} entry through the Na^{+}/Ca^{2+} exchanger. Further studies on the role of the altered sodium handling in myocardial damage in AT1aR-KO with Ald-NaCl treatment may be warranted.

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