行政院國家科學委員會專題研究計畫 成果報告

C 急性反應蛋白在 HL1 細胞株及新生心房纖維母細胞向上調控血管收縮素 II 第一型接受體(第 2 年)
研究成果報告(完整版)

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中 文 摘 要 ： 心房顫動是臨床最常見的心律不整。在一般人群的總患病率在 0.4%，隨著年齡增加，心房顫動有逐漸增加的趨勢，在 75 歲以上人群可達 10%。心房顫動會明顯增加腦中風及心臟衰竭甚至死亡的機會，因此心房顫動是消耗社會成本很重的一種心律不整的疾病。近年來有許多研究指出腎素-血管收縮素-留鹽激素系統 (renin-angiotensin-aldosterone system, RAAS)與發炎反應及 AF 有潛在的關連性。最近的研究發現，在老鼠體內，心房壓力上升時，會促使心房血管收縮素上升，進而造成心房結構重構，使心房纖維化(AF)及心房擴大。心房纖維顫動的發生和腎素-血管張力素系統有很大的關係，血管張力素可使得心房纖維顫動更容易發生及維持。當血管收縮素結合在血管收縮素 II 第一型接受體時，會促使血管收縮使血壓上升，並促進腎上腺增加留鹽激素的產生和分泌，留鹽激素亦會調控血管收縮素 II 第一型接受體的表現。本研究的目的，主要在於留鹽激素和心房纖維顫動發生機轉的關係。探討 Aldosterone 是否造成心肌細胞內 AT1； mineralocorticoid receptors (MR)及纖維化指標分子等蛋白表現量的改變，進而影響心臟之功能。在本研究報告，我們利用細胞模式，來探討留鹽激素在促進心房纖維顫動之可能機制。研究結果顯示，在培養 HL-1 心房細胞時，加入留鹽激素可以促使 MR、AT1 及纖維化指標分子蛋白表現增加，並且觀察 MAPK 活化的表現；在加入留鹽激素拮抗劑 eplerenone 或血管收縮素第一型受體阻斷劑 losartan 會抑制留鹽激素促使纖維化指標分子（COL1A, COL3A, TGF-β1, α-SMA）蛋白表現增加，並觀測到其表現是經由 MAPK 路徑調控；利用 MAPK 磷酸化抑制劑處理，發現阻斷留鹽激素路徑的活化。因此留鹽激素在心房纖維顫動病變可能扮演重要的角色，其機轉和 MAPK 之相關訊息傳導路徑有關。

中文關鍵詞： 心房顫動，留鹽激素，血管收縮素 II 第一型受體，HL-1 心房細胞

英 文 摘 要 ： Objectives To investigate the regulation of aldosterone- mineralocorticoid receptor (MR) on the expression of fibrotic marker proteins in cultured HL-1 cardiomyocytes.
Background Aldosterone is increasingly recognized for its involvement in atrial structural remodeling. However, the precise molecular mechanisms and signal pathways underlying aldosterone-induced atrial fibrosis are unknown.

Methods Western blotting was used to investigate the effects of aldosterone on the expression of MR, angiotensin II type I receptor (AT1), mitogen-activated protein kinases (MAPKs), and fibrotic marker proteins in cultured HL-1 cardiomyocytes.
Results Aldosterone concentration and time-dependently upregulated MR and AT1 expression； and time-dependently increased activation of the extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK), p38MAPK pathways, and the protein expression of collagen 1A and 3A (COL1A and COL3A), transforming growth factor (TGF)-β1, and α-smooth muscle actin (SMA). Pre-treatment with eplerenone (10-0 M) prevented the increased expression of MR, MAPK signals and the above profibrotic molecules, but amplified the increase in AT1 level stimulated by aldosterone (10- M). Pre-treatment with losartan (10-0 M) or MAPK pathway inhibitors (U0126 or SP600125) abolished aldosterone-induced MR upregulation and significantly inhibited the expression of the above fibrotic marker proteins, indicating the critical role of MR and the requirement for active AT1 in the development of aldosterone-induced atrial fibrosis.
Conclusions Elevated MR activity plays a central role in aldosterone-mediated activation of the MAPK signaling pathway and subsequent profibrotic effects in HL-1 atrial cells. MR/AT1 and the MAPK signaling pathway interact to trigger the molecular mechanism underlying the aldosterone-induced atrial fibrotic response.

英文關鍵詞： aldosterone, angiotensin, fibrosis, atrial fibrillation, signal transduction

Cross-Talk Between Mineralocorticoid Receptor/Angiotensin II Type 1 Receptor and Mitogen-Activated Protein Kinase Pathways Underlies Aldosterone-Induced Atrial Fibrotic Responses in HL-1 Cardiomyocytes

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Running title: Aldosterone-Induced Atrial Profibrosis

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Structured Abstract

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aldosterone-induced atrial fibrotic response.

Key Words: aldosterone, angiotensin, fibrosis, atrial fibrillation, signal transduction

Abbreviations Lists

AF, atrial fibrillation; AT1, angiotensin II type 1 receptor; COL,collagen; ERK, extracellular signal-regulated protein kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MR, mineralocorticoid receptor; SMA, smooth muscle actin; TGF, transforming growth factor

Introduction

Atrial fibrillation (AF) is the most common sustained arrhythmia in clinical medicine. AF is a progressive disease. Extensive evidence indicates that structural remodeling, particularly atrial fibrosis, is an important contributor to the AF substrate (1, 2). In animal models, experimentally-induced atrial fibrosis increased the likelihood of AF (3, 4). Atrial tissue samples from patients with isolated AF also showed increased fibrosis (5). Interstitial fibrosis is promoted by the increased expression of profibrotic cytokines (e.g., transforming growth factor [TGF]-β and α-smooth muscle actin [SMA]), and more pronounced simultaneous expression of collagen I and III (COL1A and COL3A) (2). It has been hypothesized that atrial fibrosis may facilitate local intra-atrial conduction block and atrial heterogeneity, thereby enhancing the propensity to AF occurrence and self-perpetuation (2, 6). However, the regulatory mechanisms and signaling pathways involved in the development of atrial fibrosis are still not completely understood.

A growing body of evidence supports the key role of the renin-angiotensin-aldosterone system in the pathogenesis of AF (7). Recent evidences suggest atrial fibrosis is promoted by enhanced extracellular signal-regulated protein kinase (ERK) and angiotensin II type 1 receptor (AT1) activation in humans and animal models of AF (6-10). Progressive interstitial changes provoked by mitogen-activated protein kinase (MAPK), coupled to the angiotensin II regulatory pathway, increase the risk of AF (7). These results suggest that the atrial renin-angiotensin system plays a major role in the development of the remodeling process in AF. Aldosterone, a well-known effector hormone of the renin-angiotensin system, has a more pro-fibrotic role than angiotensin II (11). Aldosterone causes atrial fibrosis independent of changes in wall stress or hypertension (12). Reil et al. recently demonstrated that aldosterone induces a substrate for AF with locally disturbed conduction, independent of increased atrial load and characterized by

atrial fibrosis and myocyte hypertrophy (13). Furthermore, previous experimental heart failure and AF models showed that aldosterone blockade reduces atrial fibrosis and suppresses AF (12, 14-16). Clinically, plasma aldosterone levels are raised in AF patients, and the level of mineralocorticoid receptor (MR) is higher in the atria of AF patients (17, 18). A recent genetic analysis also showed that a specific polymorphism related to increased aldosterone synthase activity predisposes heart failure patients to AF (19). Moreover, in the RALES trial, patients randomized to spironolactone had a significant reduction of both pro-collagen I and III when compared with patients receiving placebo (20). Therefore, aldosterone may contribute mechanistically to the pathogenesis of AF-promoting structural remodeling. However, the precise molecular mechanisms and signaling pathways involved are unknown. The purpose of our study was two-fold: 1) to examine the effect of aldosterone with different concentrations at different time intervals on the expression of AT1, MR, MAPK signaling molecules, and fibrosis-related molecules in cultured HL-1 atrial myocytes ; 2) to determine the involvement of AT1/MR activity and MAPK signaling pathways in the mechanism of aldosterone-induced atrial fibrosis.

Methods

This experimental protocol was approved by the Institutional Animal Care and Use Committee of the Chung Shan Medical University and complied with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication No. 3040-2, revised 1999).

HL-1 Cell Culture

The HL-1 cell line derived from adult mouse atria was obtained from National Taiwan University Hospital, Taipei (through the courtesy of Dr. Chia-Ti Tsai). The HL-1 cells were cultured essentially as described by Claycomb et al (21). Briefly, cells were maintained in

Claycomb medium (JRH Biosciences, Lenexa, KS, USA) supplemented with 2 mM L-glutamine (Gibco, Grand Island, NY, USA), 0.1 mM norepinephrine (Sigma Chemical, St. Louis, MO, USA), 10% fetal bovine serum (FBS; JRH Biosciences), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco) in 5% CO_2 at 37°C and 95% humidity. They were grown in 10-cm plates (dishes) coated with 5μg/ml fibronectin (Sigma) and 0.02% gelatin (Sigma) and until cells reached confluence. The cells were passaged every 3 days by trypsination (trypsin/EDTA, Invitrogen, Carlsbad, CA,USA), followed by addition of soybean trypsin inhibitor (Sigma). The initial plating density was in the range of 0.5–1.4 x 10^3 cells/mm^2. Dishes with an initial plating density higher than 1.4 x 10^3 cells/mm^2 are quickly overpopulated and the cells become unhealthy. The cells used for experiments were starved in Claycomb medium without FBS 48 hours after seeding or until the cultures had reached approximately 80% confluence (22). HL-1 cells were plated in 35-mm culture dishes at a density of 1×10^6 cell/dish. Prior to experiments, cells were left in serum-free medium for 24 hours, and subsequently stimulated with aldosterone or different inhibitors. This was followed by protein isolation.

Drug Treatments

After serum starvation for 24 hours, cells were treated with aldosterone (10^{-5}, 10^{-6}, 10^{-8}, 10^{-10}, 10^{-12}M) and subsequently harvested after 0, 1, 3, 6, 12 and 24 hours. Cells were pre-treated with the following drugs as indicated:

The stock solution of **Aldo**, aldosterone powder ([1 mg/ml]; Fluka, Buchs, Switzerland) was prepared by addition of DMSO (Sigma), vortexing until dissolved, and storing at –20°C until use. Aldosterone treatment was carried out by adding the stock solution (10 mM in DMSO) diluted in minimal medium of the apical and basolateral solutions to the above concentrations.

The stock solution of **Epl**, eplerenone-A (10 mM; Tocris, Avonmouth, Bristol, UK), and **Los**,

losartan A (1 mM; Fluka, Buchs, Switzerland), were prepared in DMSO according to the instructions provided with the product and diluted over 1×10^3-fold with medium immediately before use.

Protein Extracts and Western Blot Analysis

Using Western blotting, we investigated the effects of aldosterone with or without varied blockers on the expression of MR/AT1 levels, phosphorylation of different MAP kinases, and fibrosis-related proteins (TGF-β, α-SMA, COL1A and COL3A) in cultured HL-1 atrial cells.

Total protein was extracted from cells after culture under several experimental conditions using a Pro-prep protein extraction solution (iNtRON. Biotechnology, Seoul, Republic of Korea). The homogenates were centrifuged at 15,000X g for 20 min at 4°C and stored at –20°C. Protein concentrations were determined using the DC Protein Assay kit (BioRad Laboratories, Inc., Hercules, CA,USA) and 50 μg of soluble protein per sample was loaded onto 8% sodium dodecylsulfate (SDS) polyacrylamide gels, subjected to electrophoresis (SDS-PAGE), and electrotransferred to polyvinylidene difluoridep (PVDF) membranes (Millipore, Bedford, MA, USA).

The membranes were blocked with 3% bovine serum albumin (BSA) for 1 hour at room temperature and incubated with either antibody against MR (Abcam, Cambridge, MA, USA), α-SMA (Santa Cruz Biotechnology, Santa Cruz, CA, USA), TGF-β1 (Geneway Biotech, San Diego, CA, USA), COL1A (Abcam), p38α, p38 MAPK(pT180/pY182), pan-JNK/SAPK, JNK/SAPK(pT183/pY185), ERK, or ERK (pT201/pY204) (BD Biosciences, San Jose, CA, USA) at 4°C for overnight. A duplicate membrane was probed with anti-β-Actin (Geneway). The membranes were then incubated with a species-specific horseradish peroxidase-labeled secondary antibody (BD Biosciences: 1:5000) for 1 hour at 37°C. Between each step, the

membranes were thoroughly washed with Tris buffered saline Tween (TBST). Peroxidase activity was detected on ImageQuant LAS 4000 mini biomolecular imager using ECL reagents (Millipore, Billerica, MA, USA). For quantification purposes, blots were quantified using a luminescent imager (LAS-1000 Image Analyzer, Fujifilm, Berlin, Germany) and FluorChem image software (Alpha Innotech, San Leandro, CA, USA).

Removal of primary and secondary antibodies was carried out at 50°C for 30 minutes in stripping buffer (100mM β-mercapthoethanol, 2% SDS, 62.5 mM Tris-HCl [pH 6.7]). After removal of antibodies, nonspecific interactions were re-blocked by incubating in TBST, 3% BSA for 1 hour before re-probing with primary antibody.

Immunohistochemical Analysis

For immunofluorescence, HL-1 cells grown on gelatin-fibronectin coated coverslips were serum starved for 24 hours, stimulated by 1 μM aldosterone under various conditions, fixed by 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min, permeabilized in 0.5% Triton-X100 for 10 min, blocked with 1% BSA in PBS for 1 hour at room temperature, stained 2 hours at room temperature with antibody against MR (Abcam), α-SMA (Santa Cruz Biotechnology), TGF-β1 (Geneway), COL1A (Abcam) and COL3A (Santa Cruz Biotechnology) all diluted 1:100 dilution in 1% BSA in PBS, washed with PBS twice, and incubated for an additional 60 min at room temperature with goat anti-mouse IgG or goat anti-rabbit IgG conjugate with fluoresce in isothiocyanate (FITC) at 1:200 dilution in 1% BSA in PBS. Images were acquired with a DP72 digital camera and DP2-BSW microscope digital camera software. As a negative control, HL-1 cells were incubated with the secondary antibody alone. To measure staining intensity, digital images were sectioned into basal and apical compartments and density of the immunofluorescent marker staining was performed using the pixel intensity routine from

Image J software (National Institutes of Health, Bethesda, MD, USA). Uniform microscope and laser settings were used for each experimental condition. Pixel intensity changes are expressed as a percent increase or decrease, and the magnitude of these changes was statistically evaluated.

Statistical Analysis

All data are expressed as mean ± SEM of at least three independent experiments. Data were compared using Student's *t*-test for two-group data, and analysis of variance with posthoc *t*-test and corrections for *P* values was used for more than two group data. Paired *t*-test was used for comparison of protein level before and after aldosterone or the blockers. The *P*-values indicating statistical significance are given in the respective figure caption. A *P* value <0.05 was considered statistically significant.

Results

Effect of Aldosterone on MR and AT1 Expression

To evaluate the effect of aldosterone on atrial renin-angiotensin activity, we exposed HL-1 cells to aldosterone at different concentration (10^{-5}, 10^{-6}, 10^{-8}, 10^{-10}, 10^{-12}M) and for different time periods (1, 3, 6, 12 and 24 hours), and examined the protein expression of MR and AT1. As shown in Figure 1, aldosterone dose-dependently increased MR protein expression; aldosterone concentrations as low as 10^{-8} M induced a significant increase, and 24 hours of incubation with the highest concentration (10^{-5} M) induced a 2.57-fold increase ($P<0.01$). A significant increase in MR expression was seen after 1 hour exposure. Aldosterone induced an increase in AT1 protein expression in a concentration-dependent manner (Figure 1), with the maximal response (2.07-fold increase) reached at 10^{-8} M after 12 hours. A significant increase in AT1 expression was also seen after 1 hour exposure.

Subsequently, we treated HL-1 cells with a relatively high concentration (10^{-6} M) of

aldosterone, because plasma aldosterone level as high as 10^{-7} M has been reported in individuals with congestive heart failure.[23] Aldosterone significantly and time-dependently increased MR protein levels, reaching a 2-fold increase after 24 hour (Figure 2A) and significantly increased AT1 protein levels at all times (P<0.01), reaching a maximal (1.76-fold) increase after 6 hours. Aldosterone (10^{-6}M) had no effect on the expression of glucocorticoid receptor.

Effects of Aldosterone on Fibrotic Markers Expression and MAPK Phosphorylation

To evaluate the effect of aldosterone on atrial fibrosis-related signaling pathway and proteins, we exposed HL-1 cells to aldosterone (10^{-6} M) and examined the time-dependency of the aldosterone effect on the expression of profibrotic proteins, and the activation of ERK, c-Jun N-terminal kinase (JNK), and p38MAPK. As shown in Figure 2B, the expression of COL1A and COL3A proteins (the major cardiac fibrotic markers) was significantly increased up to 1.55-fold and 1.37-fold (P<0.01), respectively. TGF-β1 (a critical profibrotic factor) and α-SMA (a trans-differentiation marker) were also up-regulated 1.38-fold and 1.44-fold (P<0.01), respectively. Aldosterone significantly increased ERK, JNK, and p38MAPK phosphorylation (P<0.01) in a time-dependent manner (Figure 2C). Western blotting showed that JNK and p38 MAPK phosphorylation was induced within 1 hour and persisted for 24 hours, reaching a maximal (≈ 2-fold) at 24 hours. ERK phosphorylation was increased by 38% (P<0.01) at 12 hours and by 116% (P<0.01) at 24 hours.

Aldosterone-induced Fibrotic Cascade via MR Coupled to Functional AT1

To identify whether MR is involved in the aldosterone-induced fibrotic cascade and signaling, HL-1 cells were exposed to aldosterone (10^{-6} M) with or without pre-exposure to eplerenone (10^{-12} M and 10^{-10} M), a highly selective MR antagonist, to determine the expression

of MR and AT1, fibrosis marker proteins and ERK and JNK phosphorylation. As shown in Figure 3A, pre-treatment with eplerenone significantly inhibited the aldosterone-induced increase in MR expression but significantly augmented the aldosterone-induced increase in AT1 expression. Eplerenone (10^{-10} M) significantly reduced COL1A level and abolished aldosterone-induced phosphorylation of ERK and JNK, and eplerenone (10^{-12} M and 10^{-10} M) significantly reduced COL3A, TGF-β1 and α-SMA levels.

To investigate the effect of AT1 on the aldosterone-induced fibrotic cascade and signaling, HL-1 cells were exposed to aldosterone (10^{-6} M) with or without pre-exposure to the AT1 blocker losartan (10^{-12}M and 10^{-10}M). As shown in Figure 3B, losartan (10^{-10}M) indeed abolished the aldosterone–induced increases in MR expression and COL1A, COL3A, TGF-β1 and α-SMA levels to those comparable to the negative control levels (vehicle in the absence of aldosterone and losartan), and abolished ERK but not JNK phosphorylation. Losartan (10^{-12} M) also significantly reduced aldosterone-induced TGF-β1 and α-SMA levels (P<0.05). These results implicated AT1 in some of the signaling pathways induced by aldosterone.

Taken together, these results suggested that activation of MAPK signaling and fibrotic marker proteins in aldosterone-treated HL-1 cells is tightly regulated by cross-talk between AT1 activation and MR activation in response to aldosterone. Our data support the critical role of elevated MR activity in the aldosterone-mediated increase in fibrosis marker proteins and activation of MAPK signaling. The response of aldosterone also required the presence of activated AT1. The amelioration by losartan of aldosterone-induced atrial fibrosis seems to be partially attributable to the attenuation of aldosterone-induced MR expression.

Aldosterone-induced Fibrotic Response via MAPK-Dependent Pathways

To further investigate whether aldosterone-induced collagen synthesis is mediated by the

MAPK signaling pathway, HL-1 cells were pre-incubated with U0126 (a specific MAPKK/MEK inhibitor of ERK activation) and SP600125 (an inhibitor of the JNK phosphorylation pathway) to examine the expression of MR, AT1, and fibrotic marker proteins, and pretreated cells were compared to those not pre-treated. As shown in Figure 4, U0126 (10^{-5} M) and SP600125 (10^{-5} M) prevented the aldosterone–induced increase in COL1A, COL3A, TGF-β1 and α-SMA levels, indicating that the aldosterone-induced fibrotic response is mediated by MAPK signaling pathway. Of particular interest in our data is that pre-treatment with MAPK pathway inhibitors abolished alosterone-stimulated MR and AT1 expression. This negative feedback effect of MAPK signaling inhibitors on MR expression lends further support to the notion that elevated MR activity plays a central role in promoting the aldosterone-induced fibrotic response mediated by the MAPK signaling pathway.

Immunohistochemical Analysis

We used immunofluorescence analysis to compared the relative density of fibrosis marker proteins in aldosterone-treated HL-1 cells with or without pre-treatment with blockers to MR (eplerenone), AT1 (losartan), or MAPK signaling (U0126 or SP600125). As shown in Figure 5, the densities were significantly reduced in all cells pre-treated by various blockers compared to those treated by aldosterone only. As noted previously, the increase in MR expression stimulated by aldosterone could be abolished in all the HL-1 cells by pre-treatment with the above blockers. Taken together, our results suggested that MR/AT1 and the MAPK signaling pathway interact in a mutually cooperative manner to maintain the functional integrity of both AT1 and MR necessary for the aldosterone-induced atrial fibrotic response.

Discussion

The major findings of our study on cultured HL-1 cardiomyocytes are that (1) aldosterone

treatment upregulates AT1 expression (positive feedback) independent of angiotensin II production by atrial cells; (2) elevated MR activity plays a critical role in aldosterone-mediated activation of the MAPK signaling pathway and atrial fibrotic response; and (3) for the first time to our knowledge, treatment with AT1 blocker (losartan) and MAPK signaling inhibitors (U0126 or SP600125) downregualtes MR expression induced by aldosterone (negative feedback), thereby preventing aldosterone-induced atrial fibrotic reactions. Our study supports the idea that interaction between MR/AT1 and the MAPK pathways produces positive feedback that is important in atrial fibrosis development.

Profibrotic Effects of Aldosterone Require Functional MR and AT1 Coupling

Aldosterone has been shown to upregulate ventricular and vascular AT1 and angiotensin converting enzyme expression, indicating the deleterious cardiovascular effects of aldosterone possibly mediated by activation of the renin-angiotensin system (24-27). The aldosterone-mediated activation of AT1 in the absence of local angiotensin II production also represents an example of receptor transactivation by MR. Previous studies have shown that transactivation of MR is a critical aspect of angiotensin II-mediated cardiac or vascular signaling (28, 29). MR blockade downregulates angiotensin II-mediated signaling and reduces angiotensin II-induced end-organ damage, indicating that the effects of angiotensin II and MR activation are additive. On the other hand, the reciprocal interaction between aldosterone and AT1 has also been reported. Aldosterone upregulates the expression of angiotensin-converting enzyme and AT1, and potentiates angiotensin II-stimulated intracellular signaling and proliferation in cardiovascular diseases (24-27). Furthermore, Robert et al. demonstrated that AT1 blockade significantly prevents the aldosterone-induced cardiac collagen expression and cardiac fibrosis *in vivo* (26). Thus, aldosterone-induced cardiac fibrosis seems to be partially attributable to the

cross-talk between aldosterone and AT1, either directly or indirectly. However, mechanisms underlying this interaction remain unclear. Spironolactone, an MR antagonist, has been shown to suppress the upregulation of angiotensin converting enzyme and AT1 by aldosterone, suggesting that MR is part of a positive feedback loop for local control of the renin-angiotensin system (26, 27, 30). These results suggest that aldosterone-induced cardiac and vascular damage depends in part on activation of an angiotensin II-AT1-mediated pathway. By contrast, our study showed that pre-treatment with eplerenone (a specific MR blockade) significant enhanced aldosterone-induced AT1 upregulation and reduced MR expression in cultured HL-1 atrial cells. Discrepancies between our study and prior studies may be explained in part by the use of different cell types (atrial versus ventricular or vascular smooth muscle cells). Previous experiments have shown that aldosterone interacts with angiotesin II-AT1 signaling via an MR-independent mechanism or is mediated by a plasma membrane receptor distinct from the classical cytosolic MR first proposed by Wehling et al (31). Chai et al. showed that aldosterone potentiated the vasoconstrictor effect of angiotensin II in coronary arteries, and that this effect was not blocked by spironolactone (32). Yamada et al. further demonstrated that the vasoconstrictor effect of aldosterone is nongenomic and independent of classical MR and is not blocked by spironolactone but by AT1 blockers (33). Thus, it seemed reasonable to assume that aldosterone could transactivate AT1 independent of MR in HL-1 atrial myocytes. Our results also indicated that the aldosterone-induced activation of MAPK signaling and fibrotic marker proteins is directly mediated by MR and not by activation of AT1. However, we also showed that pre-treatment with the AT1 inhibitor, losartan, could abolish the increase in MR expression induced by aldosterone in parallel with reduction of ERK phosphorylation and fibrosis-associated proteins expression, indicating that the effects of aldosterone depend on AT1

activation. *In vitro* studies of vascular smooth cells exposed to aldosterone demonstrated increased production of reactive oxygen species, transactivation of the epidermal growth factor receptor, and activation of ERK and JNK signaling, whereas AT1 blockade inhibited these effects (28). Moreover, by using small interference RNA to knockdown AT1, Lemarié et al. demonstrated that aldosterone-induced activation of signaling pathways requires AT1 activity (34). We not only found that MR and AT1 cross talk, but most importantly, that the activation of MR by aldosterone requires a functional AT1. The present report to our knowledge is the first to demonstrate that AT1 blockade abrogated the profibrotic effect of aldosterone is probably mediated by the attenuation of aldosterone-induced MR expression. Our results shed new light on the mechanism of cross-talk between AT1 activation and MR activation. The activation of the ERK MAPK signaling pathways and profibrotic changes in response to aldosterone in cultured HL-1 atrial myocytes requires a functional AT1 coupled to elevated MR activity. These results were confirmed by pharmacological blockade of AT1 and MR, demonstrating the clinical relevance of the results from the present study.

Elevated MR Activity is Prerequisite in Aldosterone-Induced Atrial Fibrotic Response

AF is associated with substantial structural changes in atrial tissue that, to a large extent, depend on increased activity of the renin-angiotensin-aldosterone system (2, 7). Atrial fibrosis is the hallmark of structural modeling. Aldosterone is increasingly recognized to be involved in the process of atrial remodeling (12-20). Milliez et al. demonstrated that only spironolactone rather than angiotensin-converting enzyme inhibitor induced a significant decrease in atrial fibrosis in rats with heart failure following myocardial infarction, suggesting a distinct regulatory pathway and profile of atrial remodeling (14). Although previous studies support that atrial tissue is a target of aldosterone, MR-mediated signaling in atrial cells has not been systematically explored.

MR is the nuclear receptor of aldosterone and is expressed in both cardiac myocytes and fibroblasts. Eplerenone used in our study is considered to be a more selective MR antagonist without the side effects of spironolactone, including gynecomastia and breast pain, because of its low affinity for androgen and progesterone receptors. In experiments, eplerenone has been shown not merely to prevent but also reverse established cardiac fibrosis, and to suppress AF occurrence (15, 35, 36). A recent clinical trial also confirmed the added reduction in new-onset AF when eplerenone was used in patients with systolic heart failure and mild symptoms (37).

We demonstrated that aldosterone-treated cells increase their expression of MR and AT1 proteins in a concentration-dependent and time-dependent manner. Aldosterone also time-dependently induced activation of ERK, JNK, and p38MAPK signaling and expression of fibrosis marker proteins (COL1A, COL3A, TGF-β1 and α-SMA). The significant upregulation of MR, AT1, JNK, p38MAPK, and COL1A by aldosterone stimulation occurred within 1 hour. Aldosterone can exert pro-inflammatory vascular effects via MR- and ERK1/2 MAPK-dependent mechanisms (38). In accordance with previous studies showing that increased MR expression and ERK and JNK MAPK activity are involved in the process of aldosterone-induced profibrotic response, MR blockade by eplerenone and inhibition of MAPK signaling by U0126 or SP600125 reduced expression of fibrotic marker proteins in HL-1 cells to negative control levels (39). We also revealed that aldosterone induced activation of ERK and JNK MAPK through classical MR. Recent evidence indicates aldosterone has both slow, genomic and rapid, non-genomic effects, which typically observed within minutes after aldosterone stimulation and include activation of MAPK signals. Aldosterone-mediated pathologies, such as inflammation, remodeling, and endothelial dysfunction, are characterized by activation and cross-talk of genomic effects with rapid signaling pathways (28, 38, 40). Our

results do not show to what extent genomic and nongenomic mechanisms contribute to the observed effects. Thus, MR-mediated genomic actions or MR-mediated nongenomic MAPK signaling may both play a significant role in aldoserone-induced profibrotic marker expression in our cell model.

The most promising finding of the present study is that pre-treatment of cells with blockers to either receptor (losartan, eplerenone) or MAPK signaling (U0126 or SP600125) reduced levels of aldosterone-induced MR and fibrosis-related proteins, as demonstrated by Western blot and immunofluorescence analysis. Our study findings favor a growth-promoting role of MR in AF. Jaffe and Mendelsohn demonstrated that spironolactone inhibited angiotensin II-mediated MR-dependent gene expression in vascular smooth muscle cells, and highlighted a specific role for MR in regulating angiotensin II and aldosterone gene transcription (41). Increased constitutive MR signaling has been shown to promote age-associated vascular inflammation (42). Moreover, Tsai et al. demonstrated that the expression of the MR is increased in patients with AF compared to patients with sinus rhythm, indicating that MR expression is the major factor determining the effects of serum aldosterone on the atrial tissue (18). Our study further illustrated that blocking AT1 or MAPK signal pathways has a negative feedback effect (i.e., inhibits aldosterone-induced MR expression and subsequent events). Fraccarollo et al. recently demonstrated that deletion of cardiomyocyte MR improves infarct healing and prevents progressive adverse cardiac remodeling, highlighting a central role for cardiomyocyte MR in post-infarction healing and remodeling (43). The present report to our knowledge is the first to demonstrate that downregulation of MR expression seems to be the final common pathway to abolish aldosteone-mediated profibrotic effects. We demonstrate here for the first time that increased MR activity is prerequisite in the development of the atrial fibrotic response to

aldosterone. In the absence of AT1 or MR blockade, increased tissue levels of aldosterone will upregulate MR activity and feedback loops in the local renin-angiotensin system (e.g., AT1 expression), resulting in a number of vicious cycles. *In vivo*, angiotensin II and aldosterone may modulate signaling pathways and cardiovascular function either synergistically or via a cross-talk between their receptors or their respective messenger systems. In addition, recent studies have showed that angiotensin II has several pro-inflammatory properties, and the converse is also true with inflammatory stimulating angiotension II production. Thus, angiotensin II-AT1, aldosterone-MR and profibrotic pathways may interact in a mutually cooperative manner to produce positive feedback that is important in fibrosis development.

Clinical Implication

Pathophysiological synergistic effects between angiotensin II and aldosterone have been described on cardiovascular tissues (8, 28, 38). From a clinical viewpoint, our results provide evidence for a role of MR and aldosteorne signaling in the development of atrial fibrosis, expanding the mechanistic explanation of the complex interactions between the activation of MR, AT1 and their signals. Our evidence from this experimental *in vitro* study supports the beneficial role of MR blockade in conjunction with AT1 blockade or angiotensin-converting enzyme inhibitor in the prevention of AF occurrence. Clinical evidence, however, is scarce and further research is clearly warranted. Of note, EMPHASIS-HF trial has shown that eplerenone amplifies the benefits of angiotensin receptor blockers in reducing new-onset AF in patients with systolic heart failure and mild symptoms (37).

Limitation

The collagen mRNA or protein expression is often different from collagen volume fraction because collagen synthesis depends on the time course of the cardiovascular disease and the

underlying cardiovascular disease itself. Therefore, whether results in culture HL-1 cells can be readily extrapolated to atrial tissues impaired by different underlying cardiovascular diseases is unknown. In fact, it is not known whether aldosterone signaling differs between subjects with underlying chronic conditions such as ischemic cardiomyopathy, dilated cardiomyopathy, hypertension, and so on. It is also unclear whether aldosterone signaling changes with the extent of atrial remodeling. Furthermore, the role of MR blockade in different types and stages of atrial remodeling remains to be determined.

Conclusions

Aldosterone is a pathophysiological mediator of atrial fibrosis. Aldosterone mediates its fibrotic effects on HL-1 atrial cells via MR and the MAPK intracellular signaling cascade. Our study provides evidence that increased MR signaling has a central role in promoting atrial fibrotic responses, and the mutual feedback between AT1-MR and MAPK signaling activation is important for the development of these responses.

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Disclosures

None.

References

1. Allessie MA, Ausma J, Schotten U. Electrical, contractile and structural remodeling during atrial fibrillation. Cardiovasc Res 2002;54:230–46.
2. Burstein B, Nattel S. Atrial fibrosis: mechanisms and clinical relevance in atrial fibrillation. J Am Coll Cardiol 2008;51:802–9
3. Li D, Fareh S, Leung TK, Nattel S. Promotion of atrial fibrillation by heart failure in dogs: atrial remodeling of a different sort. Circulation 1999;100:87–95.
4. Verheule S, Sato T, Everett T 4th, et al. Increased vulnerability to atrial fibrillation in transgenic mice with selective atrial fibrosis caused by overexpression of TGF-beta1. Circ Res 2004;94:1458–65.
5. Frustaci A, Chimenti C, Bellocci F, Morgante E, Russo MA, Maseri A. Histological substrate of atrial biopsies in patients with lone atrial fibrillation. Circulation 1997;96:1180–4.
6. Li D, Shinagawa K, Pang L, et al. Effects of angiotensin-converting enzyme inhibition on the development of the atrial fibrillation substrate in dogs with ventricular tachypacing-induced congestive heart failure. Circulation 2001;104:2608–14.
7. Ehrlich JR, Hohnloser SH, Nattel S. Role of angiotensin system and effects of its inhibition in atrial fibrillation: clinical and experimental evidence. Eur Heart J 2006;27:512–8.
8. Goette A, Staack T, Röcken C, et al. Increased expression of extracellular signal-regulated kinase and angiotensin-converting enzyme in human atria during atrial fibrillation. J Am Coll Cardiol 2000;35:1669–77.
9. Goette A, Arndt M, Röcken C, et al. Regulation of angiotensin II receptor subtypes during atrial fibrillation in humans. Circulation 2000;101: 2678–81.

10. Boldt A, Wetzel U, Weigl J, et al. Expression of angiotensin II receptors in human left and right atrial tissue in atrial fibrillation with and without underlying mitral valve disease. J Am Coll Cardiol 2003;42:1785–92.
11. Weber KT. Aldosterone and spironolactone in heart failure. N Engl J Med 1999;341:753–5.
12. Sun Y, Ramires FJ, Weber KT. Fibrosis of atria and great vessels in response to angiotensin II or aldosterone infusion. Cardiovasc Res 1997; 35:138–47.
13. Reil JC, Hohl M, Selejan S, et al. Aldosterone promotes atrial fibrillation. Eur Heart J 2012;33:2098-108.
14. Milliez P, Deangelis N, Rucker-Martin C, et al. Spironolactone reduces fibrosis of dilated atria during heart failure in rats with myocardial infarction. Eur Heart J. 2005;26:2193–9.
15. Shroff SC, Ryu K, Martovitz NL, Hoit BD, Stambler BS. Selective aldosterone blockade suppresses atrial tachyarrhythmias in heart failure. J Cardiovasc Electrophysiol 2006;17: 534–41.
16. Zhao J, Li J, Li Y, Shan H, Gong Y, Yang B. Effects of spironolactone on atrial structural remodeling in a canine model of atrial fibrillation produced by prolonged atrial pacing. Br J Pharmacol 2010;159:1584–94.
17. Goette A, Hoffmanns P, Enayati W, Meltendorf U, Geller JC, Klein HU. Effect of successful electrical cardioversion on serum aldosterone in patients with persistent atrial fibrillation. Am J Cardiol 2001;88:906–9.
18. Tsai CT, Chiang FT, Tseng CD, et al. Increased expression of mineralocorticoid receptor in human atrial fibrillation and a cellular model of atrial fibrillation. J Am Coll Cardiol 2010;55:758–70.
19. Amir O, Amir RE, Paz H, Mor R, Sagiv M, Lewis BS. Aldosterone synthase gene

polymorphism as a determinant of atrial fibrillation in patients with heart failure. Am J Cardiol 2008;102:326–9.

20. Zannad F, Alla F, Dousset B, Perez A, Pitt B. Limitation of excessive extracellular matrix turnover may contribute to survival benefit of spironolactone therapy in patients with congestive heart failure: Insights from the Randomized Aldactone Evaluation Study (RALES). Circulation 2000;102:2700–6.
21. Claycomb WC, Lanson NA Jr, Stallworth BS, et al. HL-1 cells: A cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. Proc Natl Acad Sci 1998;95:2979–84.
22. Tsai CT, Lai LP, Hwang JJ, et al. Renin-angiotensin system component expression in the HL-1 atrial cell line and in a pig model of atrial fibrillation. J Hypertens 2008; 26:570–82.
23. Rousseau MF, Gurne O, Duprez D, et al. Beneficial neurohormonal profile of spironolactone in severe congestive heart failure: results from the RALES neurohormonal substudy. J Am Coll Cardiol 2002;40:1596–601.
24. Schiffrin EL, Franks DJ, Gutkowska J. Effect of aldosterone on vascular angiotensin II receptors in the rat. Can J Physiol Pharmacol 1985;63:1522–7.
25. Ullian ME, Schelling JR, Linas SL. Aldosterone enhances angiotensin II receptor binding and inositol phosphate responses. Hypertension 1992;20:67–73.
26. Robert V, Heymes C, Silvestre JS, Sabri A, Swynghedauw B, Delcayre C. Angiotensin AT1 receptor subtype as a cardiac target of aldosterone: role in aldosterone-salt-induced fibrosis. Hypertension 1999;33:981–6.
27. Harada E, Yoshimura M, Yasue H, et al. Aldosterone induces angiotensin converting-enzyme gene expression in cultured neonatal rat cardiocytes. Circulation

2001;104:137–9.

28. Mazak I, Fiebeler A, Muller DN, et al. Aldosterone potentiates angiotensin II–induced signaling in vascular smooth muscle cells. Circulation 2004;109:2792–800.
29. Di Zhang A, Cat AN, Soukaseum C, et al. Cross-talk between mineralocorticoid and angiotensin II signaling for cardiac remodeling. Hypertension 2008;52:1060–7.
30. Sugiyama T, Yoshimoto T, Tsuchiya K, et al. Aldosterone induces angiotensin converting enzyme gene expression via a JAK2-dependent pathway in rat endothelial cells. Endocrinology 2005;146:3900–6.
31. Wehling M, Neylon CB, Fullerton M, Bobik A, Funder JW. Nongenomic effects of aldosterone on intracellular Ca^{2+} in vascular smooth muscle cells. Circ Res 1995;76:973–9.
32. Chai W, Garrelds IM, de Vries R, Batenburg WW, van Kats JP, Danser AH. Nongenomic effects of aldosterone in the human heart: interaction with angiotensin II. Hypertension 2005;46:701–6.
33. Yamada M, Kushibiki M, Osanai T, Tomita H, Okumura K. Vasoconstrictor effect of aldosterone via angiotensin II type 1 (AT1) receptor: possible role of AT1 receptor dimerization. Cardiovasc Res 2008;79:169–78.
34. Lemarié CA, Simeone SMC, Nikonova A, et al. Aldosterone-induced activation of signaling pathways requires activity of angiotensin type 1a receptors. Circ Res 2009;105: 852–9.
35. Endemann DH, Touyz RM, Iglarz M, Savoia C, Schiffrin EL. Eplerenone prevents salt-induced vascular remodeling and cardiac fibrosis in stroke-prone spontaneously hypertensive rats. Hypertension 2004;43:1252–7.
36. Young M, Funder JW. Eplerenone, but not steroid withdrawal, reverses cardiac fibrosis in deoxycorticosterone/salt-treated rat. Endocrinology 2004;145:3153–7.

37. Swedberg K, Zannad F, McMurray JJ, et al. EMPHASIS-HF Study Investigators. Eplerenone and atrial fibrillation in mild systolic heart failure: results from the EMPHASIS-HF (Eplerenone in Mild Patients Hospitalization And SurvIval Study in Heart Failure) study. J Am Coll Cardiol 2012;59:1598–603.
38. Min LJ, Mogi M, Li JM, Iwanami J, Iwai M, Horiuchi M. Aldosterone and angiotensin II synergistically induce mitogenic response in vascular smooth muscle cells. Circ Res 2005;97:434–42.
39. Lendeckel U, Dobrev D, Goette A. Aldosterone-receptor antagonism as a potential therapeutic option for atrial fibrillation. Br J Pharmacol 2010;159:1581–3.
40. Funder JW. Minireview: aldosterone and the cardiovascular system: genomic and nongenomic effects. Endocrinology 2006;147:5564–7.
41. Jaffe IZ, Mendelsohn ME. Angiotensin II and aldosterone regulate gene transcription via functional mineralocortocoid receptors in human coronary artery smooth muscle cells. Circ Res 2005; 96:643–50.
42. Krug AW, Allenhöfer L, Monticone R, et al. Elevated mineralocorticoid receptor activity in aged rat vascular smooth muscle cells promotes a proinflammmatory phenotype via extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase ans epidermal growth factor receptor-dependent pathways. Hypertension 2010;55:1476–83.
43. Fraccarollo D, Berger S, Galuppo P, et al. Deletion of cardiomyocyte mineralocorticoid receptor ameliorates adverse remodeling after myocardial infarction. Circulation 2011;123:400–8.

Figure Legends

Figure 1. Effect of aldosterone on MR and AT1 expression. HL-1 cells were incubated with aldosterone at the indicated concentrations for the indicated times. MR and AT1 expressions were measured in cellular homogenates by Western blot analysis. Western blots contained 25 μg total protein/lane and were probed with the indicated antibody. All data were normalized to β-actin shown as a control for equal loading and blotting. Aldosterone concentration-dependently increased MR and AT1 protein expression in HL-1 cells with maximum response at 10^{-5} M and 10^{-8} M, respectively. The bar graph represents the densitometric quantification of MR and AT1 immunoreactivity, expressed as fold increase over the untreated control level at 0 hour. Values are mean±SEM (n=3, **P*<0.05, ***P*<0.01 versus vehicle).

Figure 2. Time-dependent effect of aldosterone on indicated protein expression. HL-1 cells were incubated with aldosterone (10^{-6} M) for the indicated times. The top panels in A through C show representative Western blots of the respective proteins. The bottom panels in A through C are bar graphs showing band intensities after densitometric quantification of the indicated protein bands. Each column showing the fold increase over control is the mean from three independent experiments. **Panel A:** Aldosterone time-dependently increased MR and AT1 protein expression in HL-1 cells (maximum reached at 24 and 6 hours, respectively). **Panel B:** Aldosterone time-dependently increased TGF-β1, α-SMA, COL1A and COL3A protein levels in HL-1 cells (maximum reached at 24 hours). **Panel C:** To detect activation of MAP kinases, phospho-specific antibodies that selectively recognize the active forms of p38MAPK, ERK1/2 and JNK were used. Antibodies that bind to either form of p38MAPK, ERK1/2, and JNK were used to ensure equal loading of the samples. Phosphorylated and total p38MAPK, ERK, or JNK

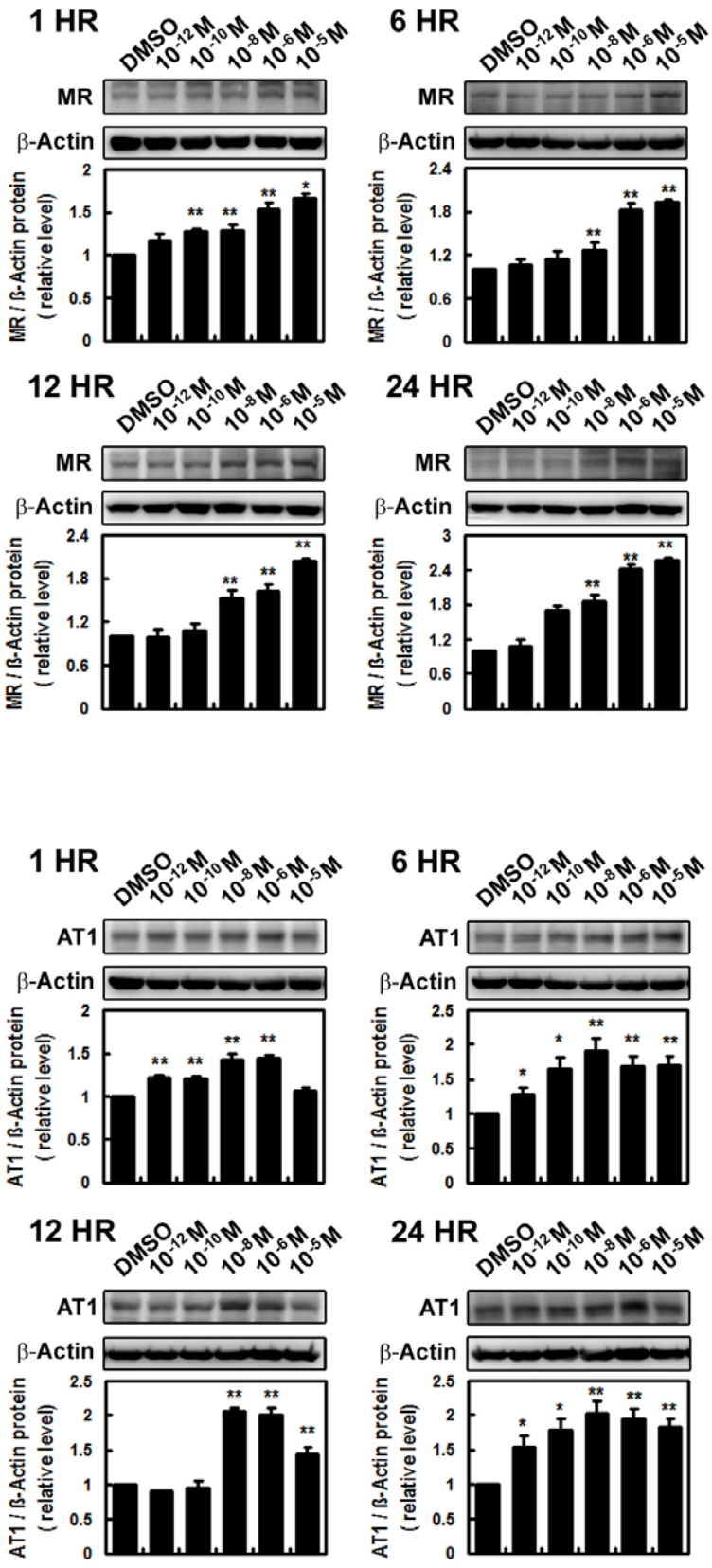
were detected by immunoblot. Aldosterone time-dependently increased p38MAPK, ERK1/2 and JNK protein levels (maximum reached at 24 hours). The data are mean ± SEM (n=3, *P<0.05, **P<0.01 vs. vehicle).

Figure 3. Panel A: Effect of eplerenone on aldosterone-stimulation of the indicated protein expressions. The HL-1 cells were pre-incubated with eplerenone (Epl; 10^{-10} or 10^{-12} M) for 30 min and then stimulated with aldosterone (10^{-6} M) for 24 hours. Pre-treatment with eplerenone significantly inhibited aldosterone-induced increases in MR, COL1A, COL3A, TGF-β1, and α-SMA expression, and phosphorylation of ERK and JNK. By contrast, eplerenone significantly augmented the aldosterone-induced increase in AT1 expression. **Panel B: Effect of losartan on aldosterone-stimulation of the indicated protein expressions.** The HL-1 cells were pre-incubated with losartan (Los; 10^{-10} or 10^{-12} M) for 30min and then stimulated with aldosterone (10^{-6} M) for 24 hours. Pre-treatment with losartan significantly inhibited aldosterone-induced increases in MR, COL1A, COL3A, TGF-β1, and α-SMA expression, and phosphorylation of ERK. The data are means ± SEM (n=3, *P<0.05, **P<0.01 vs. control) from three independent experiments.

Figure 4. Effects of ERK or JNK pathway blockade on aldosterone-stimulation of the indicated protein expressions. After 20 hours in serum-free medium, cells were pre-incubated with U0126 (10^{-5} M) or SP600125 (10^{-5} M) or DMSO as a vehicle for 2 hour and then treated with 10^{-6} M aldosterone for 24 hours. Pre-treatment with U0126 and SP600125 indeed abolished ERK and JNK activation, respectively, thereby significantly preventing aldosterone–induced increase of the levels of COL1A, COL3A, TGF-β1, and α-SMA. Notably, pre-incubating with MAPK pathway inhibitors abolished aldosteron-stimulated MR and AT1 expression. Data are mean ± SEM (n=3, *P<0.05, **P<0.01 vs. control) from three independent experiments.

Figure 5. Immunohistochemistry assay of aldosterone-treated HL-1 cells. Cells were pre-treated with inhibitors as indicated (Epl, Los, U0126 and SP600125) or vehicle, and then stimulated with aldosterone (10^{-6} M) for 24 hours. HL-1 cells were fixed and subjected to immunostaining with anti-COL1A, anti-COL3A, anti-TGF-β1, or anti-α-SMA antibody. Pixel intensity of the cells displaying fluoresces accumulation was measured. All indicated fibrotic marker protein densities were significantly reduced in all the cells pre-treated by various blockers compared to those treated by aldosterone only. Values are the arithmetic means of at least three independent experiments. The percentage of fluoresces cells was calculated per condition as described in Methods (n >100, **P*<0.01 vs. control).

Fig.1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10-10 M
10-8 M
10-6 M
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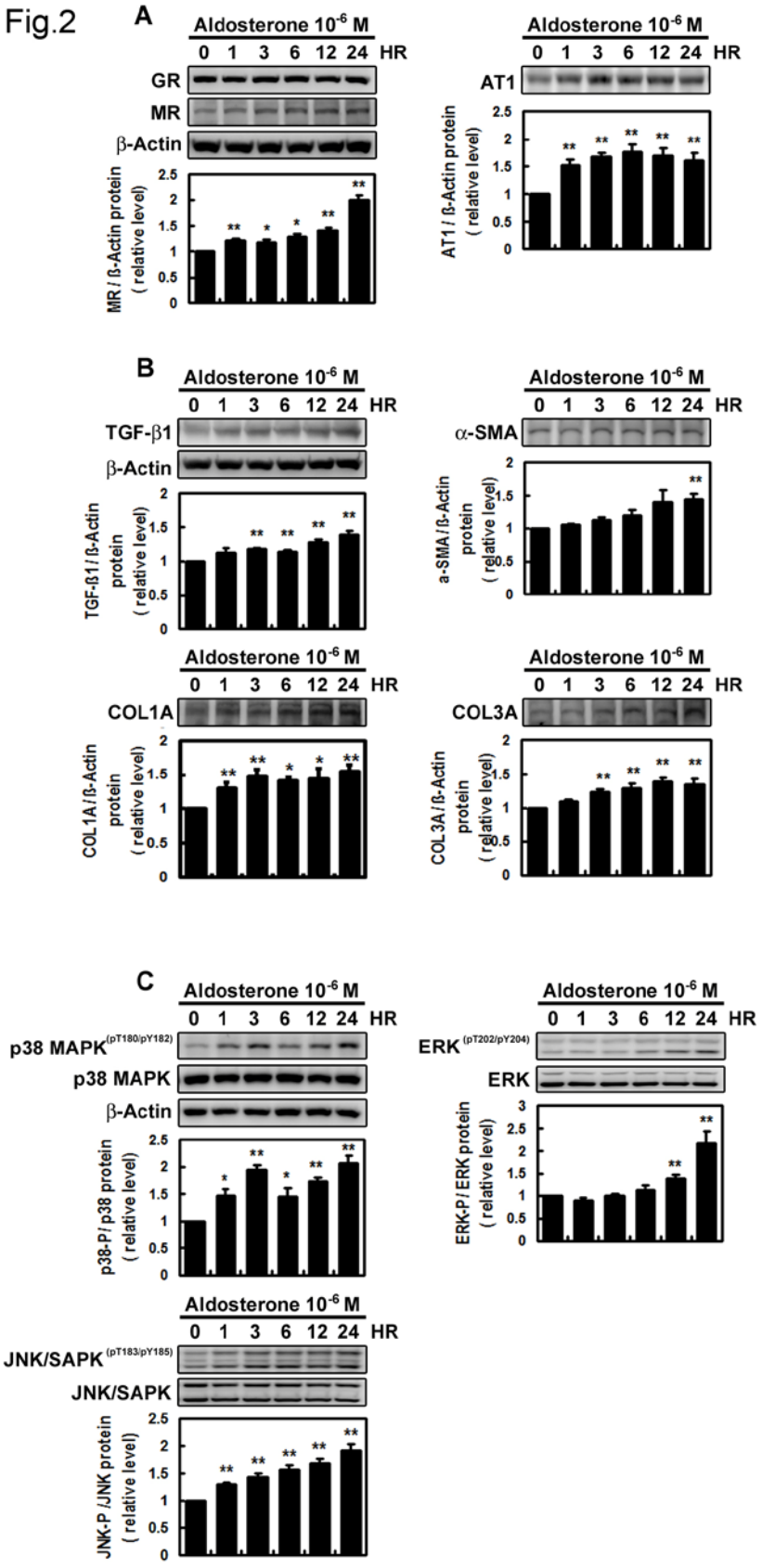
β-Actin
MR / ß-Actin protein (relative level)
AT1
AT1 / ß-Actin protein (relative level)

Fig.2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GR
MR
β-Actin
MR / β-Actin protein (relative level)
AT1
AT1 / β-Actin protein (relative level)
B
TGF-β1
β-Actin
TGF-β1 / β-Actin protein (relative level)
α-SMA
a-SMA / β-Actin protein (relative level)
COL1A
COL1A / β-Actin protein (relative level)
COL3A
COL3A / β-Actin protein (relative level)
C
p38 MAPK (pT180/pY182)
p38 MAPK
β-Actin
p38-P / p38 protein (relative level)
ERK (pT202/pY204)
ERK
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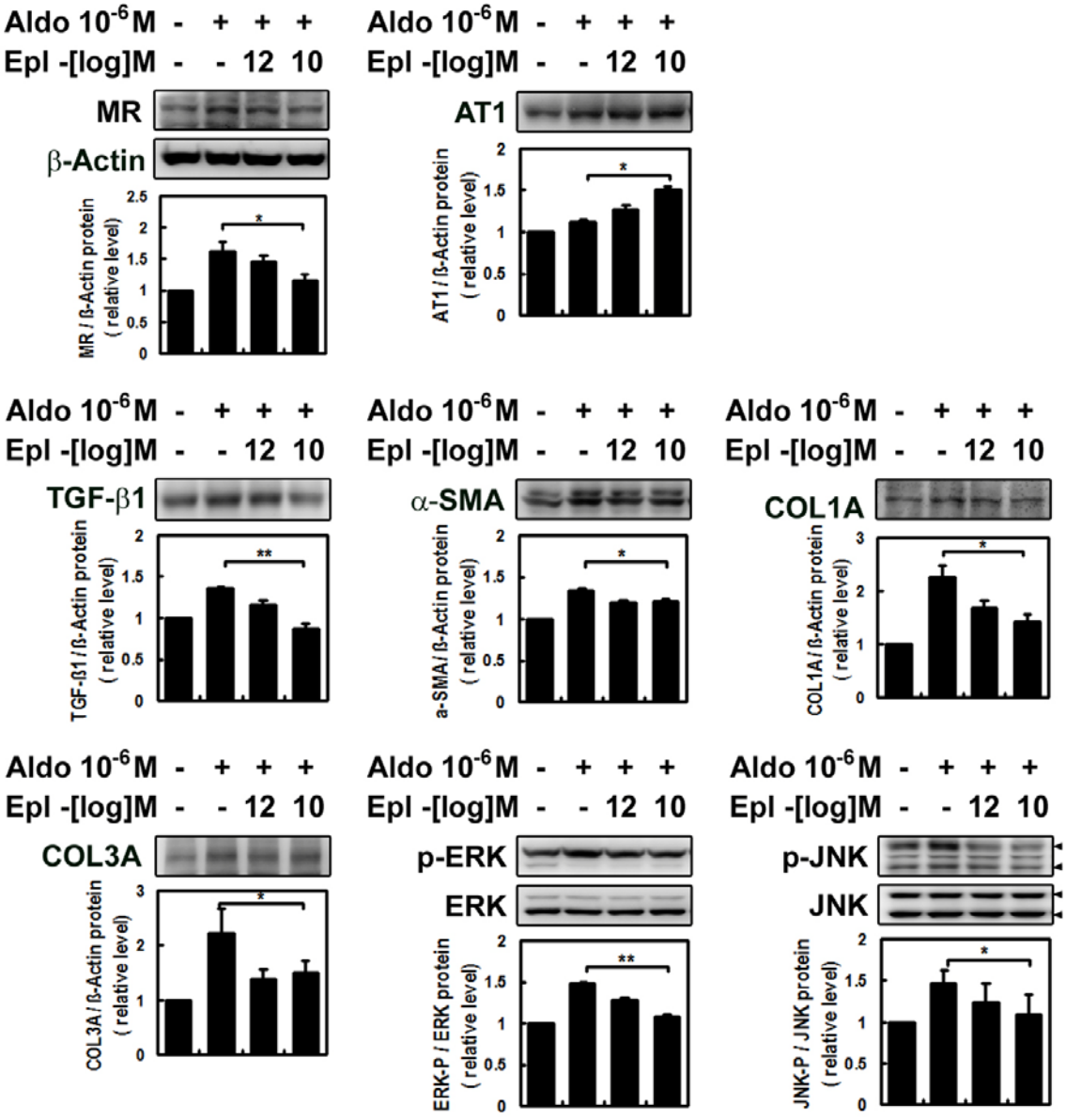
JNK/SAPK
JNK-P / JNK protein (relative level)

Fig.3

A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Epl -[log]M - - 12 10
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β-Actin
MR / β-Actin protein (relative level)
AT1
AT1 / β-Actin protein (relative level)
TGF-β1
TGF-β1 / β-Actin protein (relative level)
α-SMA
a-SMA / β-Actin protein (relative level)
COL1A
COL1A / β-Actin protein (relative level)
COL3A
COL3A / β-Actin protein (relative level)
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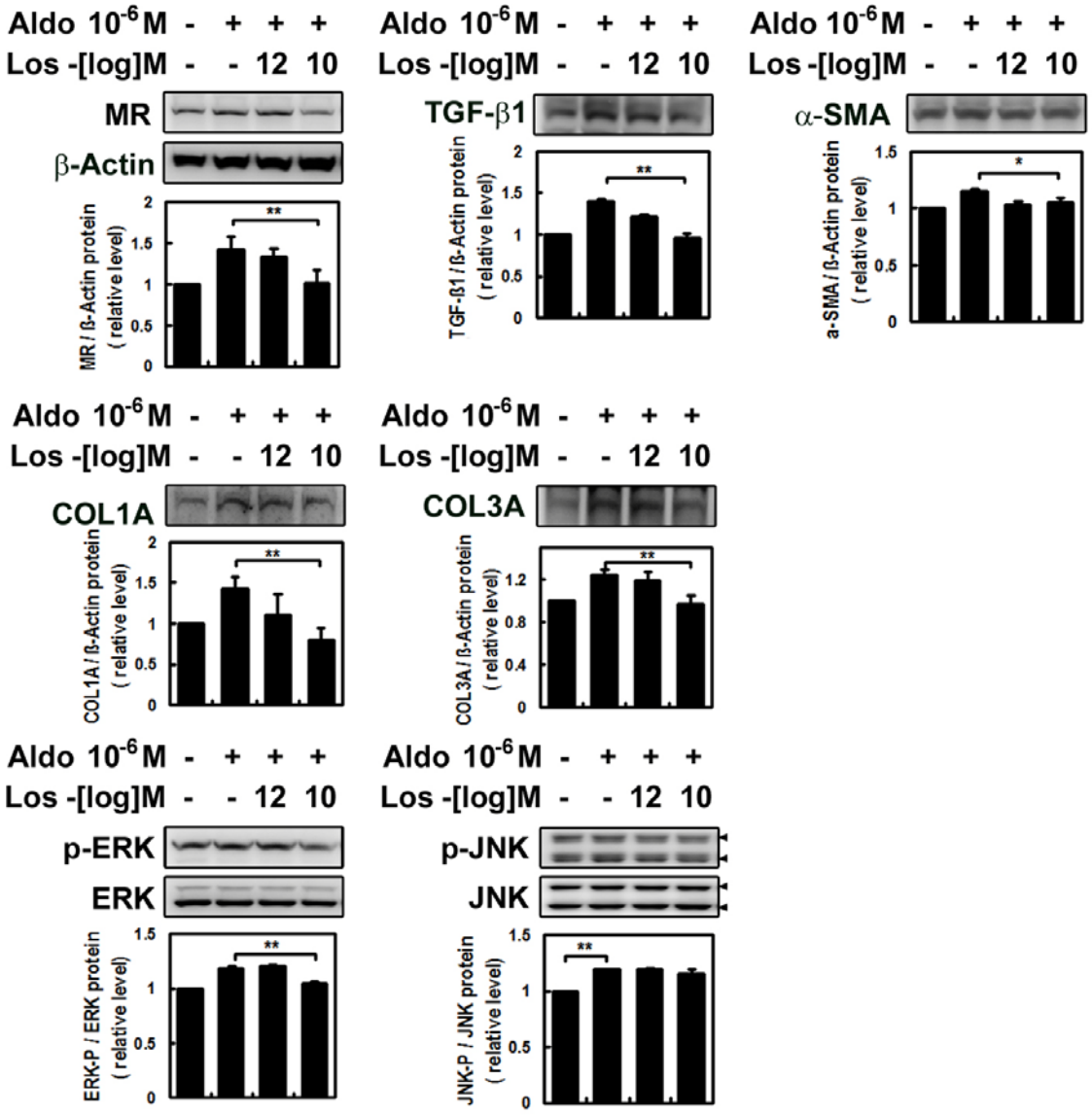
JNK
JNK-P / JNK protein (relative level)

Fig.3

B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α-SMA
a-SMA / β-Actin protein (relative level)
COL1A
COL1A / β-Actin protein (relative level)
COL3A
COL3A / β-Actin protein (relative level)
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JNK
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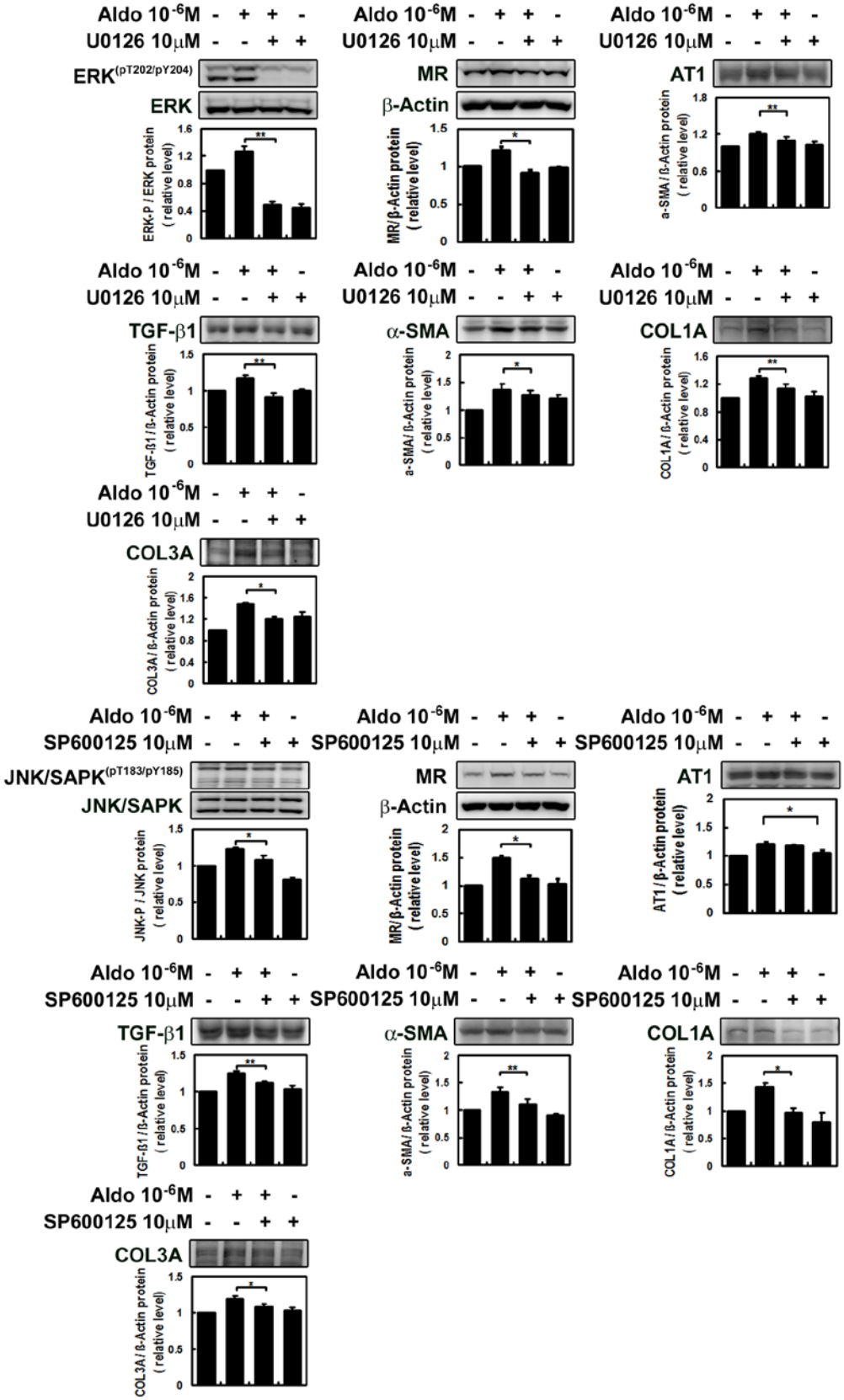
Fig.4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U0126 10μM - - + +
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MR
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MR/β-Actin protein (relative level)
AT1
a-SMA / β-Actin protein (relative level)
TGF-β1
TGF-β1 / β-Actin protein (relative level)
α-SMA
a-SMA/ β-Actin protein (relative level)
COL1A
COL1A / β-Actin protein (relative level)
COL3A
COL3A / β-Actin protein (relative level)
SP600125 10μM - - + +
JNK/SAPK(pT183/pY185)
JNK/SAPK
JNK-P / JNK protein (relative level)
MR/β-Actin protein (relative level)
AT1 / β-Actin protein (relative level)
TGF-β1 / β-Actin protein (relative level)
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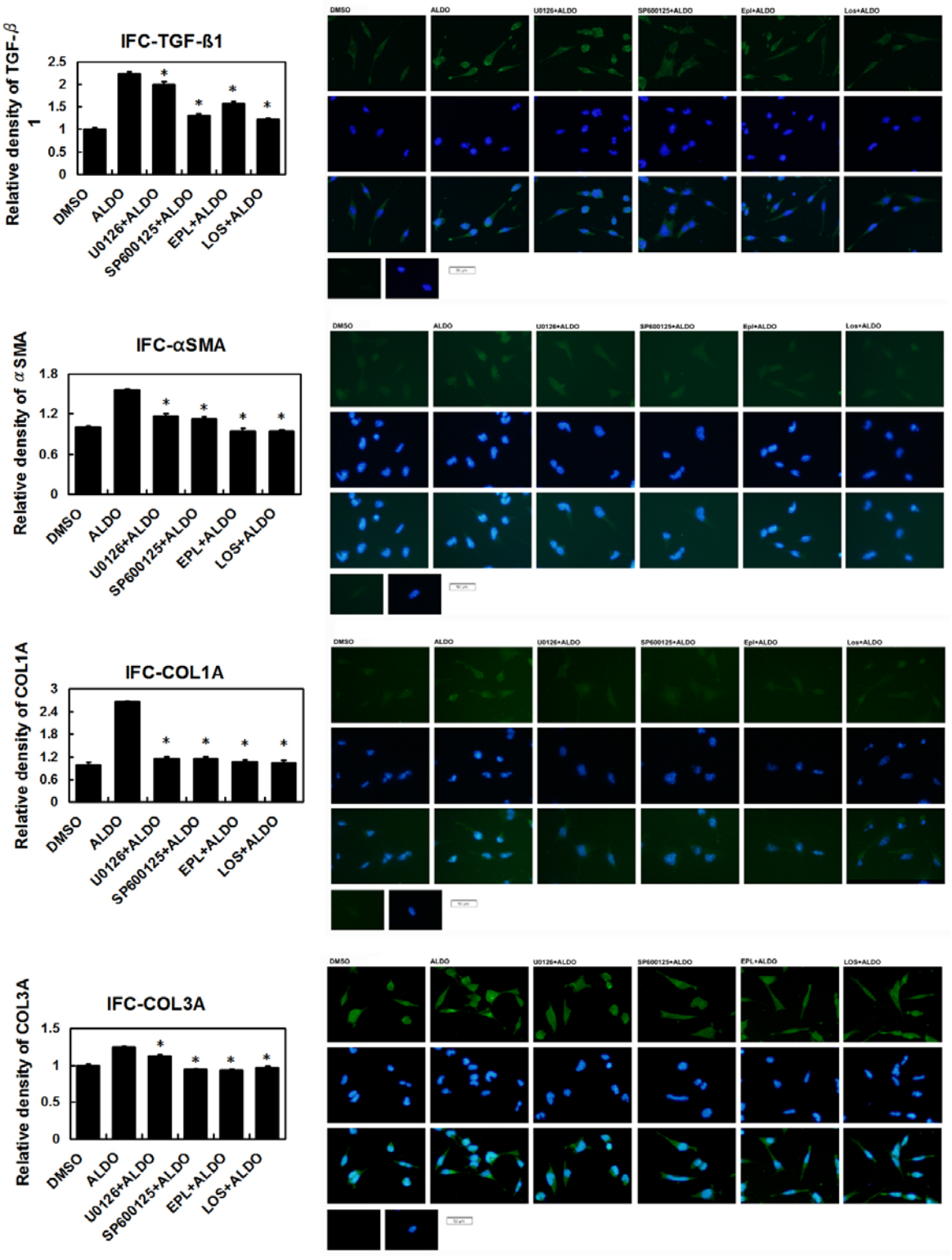
COL1A / β-Actin protein (relative level)
COL3A / β-Actin protein (relative level)

Fig.5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EPL+ALDO
LOS+ALDO
IFC-αSMA
Relative density of α SMA
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1.2
0.6
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IFC-COL1A
Relative density of COL1A
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2.4
1.8
1.2
0.6
0
IFC-COL3A
Relative density of COL3A
1.5
1
0.5
0

國科會補助計畫衍生研發成果推廣資料表

日期:2012/10/15

國科會補助計畫	計畫名稱: C急性反應蛋白在HL1細胞株及新生心房纖維母細胞向上調控血管收縮素II第一型接受體
	計畫主持人: 翁國昌
	計畫編號: 99-2314-B-040-020-MY2　　學門領域: 心胸內科

無研發成果推廣資料

99 年度專題研究計畫研究成果彙整表

計畫主持人：翁國昌	計畫編號：99-2314-B-040-020-MY2
計畫名稱：C 急性反應蛋白在 HL1 細胞株及新生心房纖維母細胞向上調控血管收縮素 II 第一型接受體	

成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數(含實際已達成數)	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%		
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（本國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		
國外	論文著作	期刊論文	0	0	100%	篇	
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		研討會論文	0	0	100%		
		專書	0	0	100%	章/本	
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（外國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		

其他成果 (無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。)	

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數		

國科會補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

■達成目標

□未達成目標（請說明，以 100 字為限）

□實驗失敗

□因故實驗中斷

□其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形：

論文：□已發表 ■未發表之文稿 □撰寫中 □無

專利：□已獲得 □申請中 ■無

技轉：□已技轉 □洽談中 ■無

其他：(以 100 字為限)

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）(以 500 字為限)

心房顫動是臨床最常見的心律不整。在一般人群的總患病率在 0.4%，隨著年齡增加，心房顫動有逐漸增加的趨勢，在 75 歲以上人群可達 10%。心房顫動會明顯增加腦中風及心臟衰竭甚至死亡的機會，因此心房顫動是消耗社會成本很重的一種心律不整的疾病。近年來有許多研究指出腎素-血管收縮素-留鹽激素系統 (renin-angiotensin-aldosterone system, RAAS)與發炎反應及 AF 有潛在的關連性。最近的研究發現，在老鼠體內，心房壓力上升時，會促使心房血管收縮素上升，進而造成心房結構重構，使心房纖維化(AF)及心房擴大。心房纖維顫動的發生和腎素-血管張力素系統有很大的關係，血管張力素可使得心房纖維顫動更容易發生及維持。當血管收縮素結合在血管收縮素 II 第一型接受體時，會促使血管收縮使血壓上升，並促進腎上腺增加留鹽激素的產生和分泌，留鹽激素亦會調控血管收縮素 II 第一型接受體的表現。本研究的目的，主要在於留鹽激素和心房纖維顫動發生機轉的關係。探討 Aldosterone 是否造成心肌細胞內 AT1； mineralocorticoid receptors (MR)及纖維化指標分子等蛋白表現量的改變，進而影響心臟之功能。在本研究報告，我們利用細胞模式，來探討留鹽激素在促進心房纖維顫動之可能機制。研究結果顯示，在培養 HL-1 心房細胞時，加入留鹽激素可以促使 MR、AT1 及纖維化指標分子蛋白表現增加，並且觀察 MAPK 活化的表現；在加入留鹽激素拮抗劑 eplerenone 或血管收縮素第一型受體阻斷劑 losartan 會抑制留鹽激素促使纖維化指標分子（COL1A, COL3A, TGF-β1, α-SMA）蛋白表現增加，並觀測到其表現是經由 MAPK 路徑調控；利用 MAPK 磷酸化抑制劑處理，發現阻斷留鹽激素路徑的活化。因此留鹽激素在心房纖維顫動病變可能

扮演重要的角色，其機轉和 MAPK 之相關訊息傳導路徑有關。