Aldosterone augments endothelin-1-induced cardiac myocyte hypertrophy with the reinforcement of the JNK pathway

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Abstract Aldosterone is thought to regulate cardiac work independently of sodium retention, though the mechanisms remain to be known. In the present study, we have demonstrated that aldosterone reinforces endothelin-mediated cardiac hypertrophy with the increase in cell surface area and upregulation of the transcripts characteristic of hypertrophy. We have also shown that aldosterone augments c-Jun N-terminal kinase activation induced by endothelin-1. Taken together, it is suggested that aldosterone modulates cardiac hypertrophy, at least partially, synergistically with extracellular signals that have been shown to be involved in cardiac remodeling.

Key words: Aldosterone; Hypertrophy; Remodeling; Signal transduction; Endothelin-1

1. Introduction

Cardiac hypertrophy is an adaptive response to pathological conditions, including myocardial infarction, genetic mutations of sarcomeric proteins, and hypertension. Various kinds of hormones, growth factors and cytokines play critical roles in cardiac hypertrophy, leading to cardiac remodeling characterized by cardiac myocyte loss and interstitial fibrosis.

As aldosterone (Aldo) receptor is highly expressed in the heart [1,2], Aldo has been believed to function as a modulator of cardiac work. A clinical study revealed that left ventricular mass index is correlated with serum Aldo levels in patients with hypertension [3], suggesting that Aldo transduces hypertrophic signals synergistically with other signals which are involved in cardiac hypertrophy.

Accumulating evidence has revealed that endothelin-1, originally isolated as a vasoconstrictor [4], contributes to the development of cardiac hypertrophy both in vitro and in vivo [5]. In addition, endothelin-1 is known to be pathophysiologically involved in cardiovascular diseases such as failing heart, considering that the inhibition of endothelin-1 prevents the progression of heart failure [6–8]. Collectively, it could be concluded that endothelin-1 functions as a modulator of cardiac remodeling.

In the present study, we explored the crosstalk between Aldo and endothelin-1 signals in cardiac hypertrophy by analyzing the morphological changes of cardiomyocytes and expression of skeletal actin [9] and brain natriuretic peptide (BNP) [10], molecular markers of cardiac hypertrophy. And we have demonstrated that Aldo augments endothelin-1-induced JNK activation. These data suggest that Aldo directly modulates cardiac remodeling, independently of sodium metabolism.

2. Materials and methods

2.1. Cell culture

Primary cultures of neonatal cardiac myocytes were prepared as described previously [11]. Briefly, the ventricles of the hearts from 1-day-old Wistar rats were treated with trypsin and collagenase. Isolated cells were collected and resuspended in M-199 containing 10% calf serum (CS). Cultures were enriched with myocytes by preplating for 60 min to deplete the non-myocyte population. Non-attached cells were plated on 60-mm plastic culture dishes at a concentration of 1×10^6 cells/well and cultured in M-199 containing 10% CS for 24 h. Immunofluorescent analysis with anti-sarcomeric α-actinin revealed that >90% of cultured cells consist of cardiac myocytes (not shown). After incubated in M-199 plus 10% CS for 24 h, cells were cultured in CS-depleted M-199. All assays were performed in serum-depleted condition.

Cells were pretreated with or without Aldo (Sigma) for 15 h, followed by stimulation with endothelin-1 (Peptide Institute) for the indicated time in the presence of the same concentration of Aldo.

To measure the cell surface areas, light photomicrographs of beating cells (250–300 cells from six visual fields) were obtained by an Olympus IMT-2 microscope and cell surfaces were traced by using Macscope program.

2.2. Northern blot analysis

The probes for BNP, skeletal actin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were kindly gifted by Dr. K.R. Chien (University of California). Northern blot analyses were performed as described previously [11]. Briefly, 5 μg of total RNA, prepared by the acid guanidinium isothiocyanate–phenol–chloroform method [12], was size-fractionated and blotted onto a nylon membrane (Hybond N+, Amersham Pharmacia Biotech). After 3 h prehybridization, the membranes were hybridized with the probes labeled with ^{32}P. After membranes were washed, autoradiography was performed at −80°C. The intensity of the bands for BNP and skeletal actin mRNA was measured and normalized by the intensity of the band for GAPDH mRNA by using densitometry.

2.3. Western blot analysis

After pretreatment with Aldo followed by 15 min stimulation with endothelin-1, cells were washed with ice cold phosphate-buffered saline and lysed with SDS–PAGE sample solution (200 μl/60-mm dish). Cell lysates were boiled for 5 min and proteins were separated on SDS–PAGE and blotted onto a polyvinylidene difluoride membrane. After blocked in TBS-T containing 2% skim milk, membranes were probed with antibodies specific for phospho-extracellular signal-regu-

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lated kinase (ERK) (New England BioLabs), p38 mitogen-activated protein kinases (MAPK) (New England BioLabs), or c-Jun NH_2-terminal kinase (JNK) (Promega). To show the efficacy of the transfer, the membranes were reprobed with anti-ERK, -p38 MAPK, or -JNK antibody (Santa Cruz Biotechnology). The ECL system (Amersham) was used for the detection.

2.4. Statistical analysis

Data are expressed as mean ± S.E.M. Statistical analysis was performed by *t*-test or ANOVA for multiple comparisons.

3. Results and discussion

3.1. Aldo augments endothelin-1-induced hypertrophy in cultured cardiac myocytes

To analyze crosstalk between Aldo and endothelin-1 in cardiac hypertrophy, we stimulated cultured cardiac myocytes with endothelin-1 in the presence or absence of Aldo. Fig. 1A shows representative phase contrast micrographs of cardiomyocytes. To quantify hypertrophic effects, we measured the cell surface area as described in Section 2. As shown in Fig. 1B, endothelin-1 alone increased the cell surface area as reported previously [9]. Though Aldo alone did not show any effects, cells costimulated with Aldo and endothelin-1 showed a more marked increase in surface area, compared with those treated with endothelin-1 alone. Furthermore, pretreatment with various concentration of Aldo revealed the dose dependence of the increase in surface area as shown in Fig. 1C. Surface area was significantly increased when pretreated with 100 nM Aldo. These data suggest that Aldo modulates cardiac hypertrophy, synergistically with endothelin-1.

In order to clarify the positive effects of Aldo on hypertrophic phenotype, we examined the expression of transcripts characteristic of cardiac hypertrophy. Cells were stimulated with or without endothelin-1 in the presence or absence of Aldo. Total RNA was Northern-blotted for BNP and skeletal actin mRNA as hypertrophic markers. As shown in Fig. 2A, Aldo augmented the upregulation of BNP and skeletal actin mRNA by endothelin-1. The expression of GAPDH mRNA was not affected by Aldo. To confirm this effect, we quantified the band intensity of BNP and skeletal actin mRNA normalized by that of GAPDH. As shown in Fig. 2B, the endothelin-1-induced increase in BNP and skeletal actin mRNA was significantly enhanced by Aldo. Next we examined the dose-dependent effect of Aldo on cardiac hypertrophy in the presence of endothelin-1. Quantification of the bands for BNP and skeletal actin mRNA intensities were shown in Fig. 2C. Aldo enhanced endothelin-1-induced hypertrophy in a dose-dependent manner. Of note, Aldo has a synergistic effect with endothelin-1 on cardiac hypertrophy under its physiological conditions.

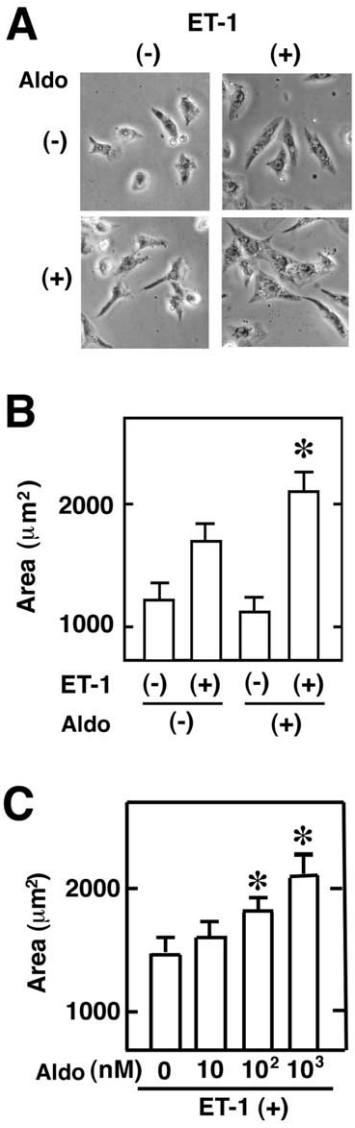

Fig. 1. Pretreatment of Aldo increases cell surface area in endothelin-1 (ET-1)-induced cardiac myocyte hypertrophy. A: Cardiomyocytes were stimulated with (+) or without (−) 10 nM ET-1 in the presence (+) or absence (−) of 1 μM Aldo for 24 h. Beating cells were identified as cardiomyocytes and examined with an Olympus IMT-2 microscope. Representative light photomicrographs of cardiac myocytes are shown. B: Cells were cultured under the conditions as described in A. Cardiomyocytes (250–300 cells for each condition) were traced and cell surface areas were measured using the Macscope program. $*P < 0.05$ versus endothelin-1 alone. C: Cells were cultured at indicated concentrations of Aldo in the presence of 10 nM ET-1. Cell surface areas were measured as described in B. $*P < 0.05$ versus endothelin-1 alone.

3.2. Treatment with aldosterone reinforced phosphorylation of JNK

In cardiac myocytes, previous studies have reported that endothelin-1 transduces hypertrophic signals through ERK, p38 MAPK, and JNK [13,14]. To explore the molecular mechanisms of positive effects of Aldo on endothelin-1-mediated cardiac hypertrophy, we analyzed the signaling pathway activated by endothelin-1. After stimulation with endothelin-1 with or without pretreatment of Aldo, activation of MAPKs was analyzed. As shown in Fig. 3A, endothelin-1 alone activated ERK and JNK while p38 MAPK was activated to a lesser extent. Aldo augmented JNK activation by endothelin-1, but not ERK or p38 MAPK. We quantified the band intensity of phosphorylated JNK normalized by that of total JNK. As shown in Fig. 3B, endothelin-1 alone significantly activated JNK as reported previously [13], interestingly, endothelin-1-mediated JNK activation was significantly enhanced by Aldo.

Though Aldo binds to glucocorticoid receptor (GR) as well as mineralocorticoid receptor, it is unlikely that Aldo augmented endothelin-1-mediated hypertrophy through GR, because of the low affinity of Aldo to GR. The K_d value of Aldo

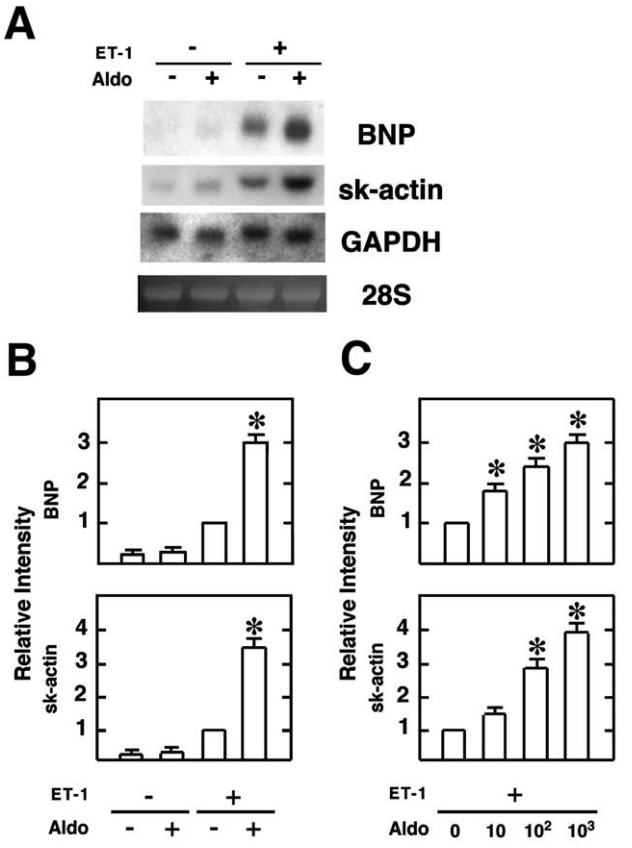

Fig. 2. Aldo enhances BNP and skeletal actin mRNA induction by endothelin-1 (ET-1): A. Cardiomyocytes were stimulated with (+) or without (−) 10 nM ET-1 in the presence (+) or absence (−) of 1 μM Aldo for 24 h. The expression of BNP, skeletal actin (sk-actin), and GAPDH mRNA was examined by Northern blot analyses. B: Relative intensity of the bands for BNP and skeletal actin mRNA were assessed as the ratio to the intensity for GAPDH. The results were expressed as relative intensity over ET-1 alone. $*P<0.05$ versus ET-1 alone. C: Cells were pretreated with 0, 10, 10^2, or 10^3 nM Aldo, followed by stimulation with 10 nM ET-1. The expression of BNP, sk-actin, and GAPDH mRNA was examined by Northern blot analyses. Relative intensity of the bands for BNP and skeletal actin mRNA was assessed as the ratio to the intensity for GAPDH. The results are expressed as relative intensity over 0 nM Aldo. $*P<0.05$ versus 0 nM Aldo.

to GR is more than 300 nM in vitro [15]. However, Aldo-induced cardiomyocyte hypertrophy is realized at 10–100 nM of Aldo (Figs. 1C and 2C). Interestingly, in the heart, the Aldo concentration is reported to be about 16 nM [16], 17-fold higher than the mean plasma level, possibly because of the slower degradation in the heart. Taking account of its paracrine effect, the data shown in this study could be physiologically suggestive.

Previously it was revealed that Aldo induces cardiac hypertrophy under high glucose culture [17]. Interestingly, high glucose culture also enhances JNK activity [18], similar to endothelin-1 stimulation, proposing the hypothesis that JNK plays an important role in Aldo-mediated cardiac hypertrophy. The causality has not been substantially documented because a JNK-specific inhibitor is not available. However, this hypothesis was supported by the experiment with BAPTA-AM. Interestingly, it is revealed, using BAPTA-AM, that angiotensin II, an agonist of the seven-transmembrane receptor similar to endothelin-1, stimulates calcium-dependent activation of JNK [19]. It is also reported that intracellular increase of calcium ion is required for JNK-mediated cardiac hypertrophy by angiotensin II [20]. Importantly, BAPTA-AM inhibited hypertrophic phenotype modulation induced by co-stimulation with endothelin-1 and Aldo, associated with the inhibition of JNK activity (not shown). Taken together with the previous report that JNK is a critical regulator of endothelin-1-induced cardiac hypertrophy [13], it could be supposed that Aldo augments endothelin-1-mediated cardiac hypertrophy, at least partially, by reinforcing JNK activation.

Pathophysiologically, left ventricular mass index is correlated with serum Aldo levels in patients with hypertension [3]. Moreover, Aldo antagonists are reported to decrease left ventricular mass index [21,22]. These studies suggest that Aldo contributes to pathological hypertrophy in vivo, though further studies are required to reveal the molecular mechanism. Interestingly, subcutaneous administration of Aldo upregulates endothelin-1, leading to cardiac remodeling [23,24]. Combined with the present study, it is possible that Aldo

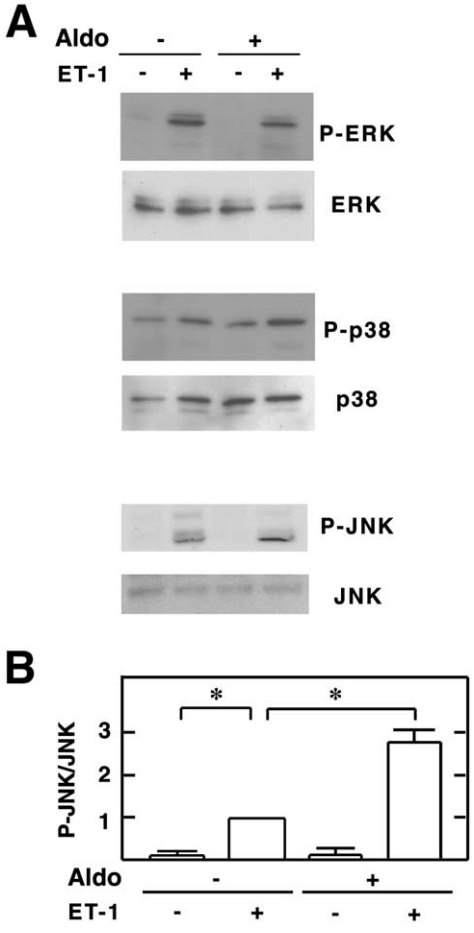

Fig. 3. Treatment with Aldo reinforces phosphorylation of JNK. A: After pretreatment with (+) or without (−) Aldo (1 μM) for 15 h, cells were stimulated with endothelin-1 (ET-1) for 15 min. Cell lysates were immunoblotted, as described in Section 2, using antibody against phospho-specific ERK1/2 (P-ERK), p38 MAPK (P-p38), or JNK (P-JNK). Membranes were reprobed with anti-ERK1/2 (ERK), -p38 (p38), or -JNK (JNK) antibody to show the efficacy of blotting. Representative data are shown. B: Relative intensity of the bands for phosphorylated JNK was assessed as the ratio to the intensity for bands for total JNK. The results are expressed as relative intensity over ET-1 alone. $*P<0.05$ versus ET-1 alone.

may not only augment the effect of endothelin-1 but increase endothelin-1 concentration, suggesting that Aldo may promote cardiac hypertrophy in vivo both by upregulating endothelin-1 and by reinforcing the hypertrophic effects of endothelin-1.

In summary, we showed that activation of the Aldo system leads to the augmentation of endothelin-1-mediated cardiac hypertrophy with the reinforcement of JNK activation. This is the first demonstration of crosstalk between the Aldo system and endothelin-1 signals in cardiac hypertrophy.

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