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The novel angiotensin II type 1 receptor (AT1R)-associated protein ATRAP downregulates AT1R and ameliorates cardiomyocyte hypertrophy

Yutaka Tanaka[a], Kouichi Tamura[a,*], Yuichi Koide[a], Masashi Sakai[a], Yuko Tsurumi[a], Yoshihiro Noda[b], Masanari Umemura[a], Tomoaki Ishigami[a], Kazuaki Uchino[a], Kazuo Kimura[a], Masatsugu Horiuchi[c], Satoshi Umemura[a]

[a] *Department of Medical Science and Cardiorenal Medicine, Yokohama City University Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan*
[b] *Laboratory Animal Facility, Yokohama City University Graduate School of Medicine, Yokohama 236-0004, Japan*
[c] *Department of Medical Biochemistry I, Ehime University School of Medicine, Shigenobu 791-0295, Japan*

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Abstract **Activation of angiotensin II (Ang II) type 1 receptor (AT1R) signaling is reported to play an important role in cardiac hypertrophy. We previously cloned a novel molecule interacting with the AT1R, which we named ATRAP (for Ang II type 1 receptor-associated protein). Here, we report that overexpression of ATRAP significantly decreases the number of AT1R on the surface of cardiomyocytes, and also decreases the degree of p38 mitogen-activated protein kinase phosphorylation, the activity of the c-fos promoter and protein synthesis upon Ang II treatment. These results indicate that ATRAP significantly promotes downregulation of the AT1R and further attenuates certain Ang II-mediated hypertrophic responses in cardiomyocytes.**

Keywords: Angiotensin II; Angiotensin II type 1 receptor; Downregulation; Signal transduction; Hypertrophy; Cardiomyocyte

1. Introduction

The renin–angiotensin system exerts a major influence on blood pressure along with sodium and the extracellular fluid balance through the generation of angiotensin II (Ang II), a key regulator of cardiovascular homeostasis, and has been implicated in the pathogenesis of cardiovascular diseases [1]. Cardiac hypertrophy is an adaptive response to both hemodynamic and non-hemodynamic stimuli, such as hypertension and myocardial infarction, and is a major risk factor for heart failure and death [2]. Previous studies have reported that angiotensin-converting enzyme (ACE) inhibitors both prevent progression and induce regression of cardiac hypertrophy in hypertensive patients [3] as well as in experimental animal models [4,5], and it has been shown that components of the renin–angiotensin system, including angiotensinogen and ACE, are upregulated in the hypertrophied heart [6,7]. These findings suggest that the renin–angiotensin system plays a role in the development of cardiac hypertrophy leading to heart failure [8].

Most of the known actions of Ang II are mediated by the Ang II type 1 receptor (AT1R) [1]. The AT1R is a member of the superfamily of G protein-coupled receptors and activates G proteins through regions of the third intracellular loop and the intracellular carboxy-terminal (C-terminal) tail of the receptor [9,10]. Previous mutation analysis has revealed that the C-terminal cytoplasmic end is involved in the control of AT1R internalization independently of G protein coupling [11]. Serial deletions of the C-terminal tail lead to a reduction in internalization and a decrease in the coupling to Gq proteins [12]. Although the molecular mechanisms behind the control of these processes are not yet completely understood, they could well involve direct interaction of the AT1R with different proteins as effectors. This would suggest that the C-terminal tail of the AT1 receptor plays an important role in linking receptor-mediated signal transduction to certain specific biological response to Ang II, including cardiac hypertrophy.

Employing a yeast two-hybrid screening system, we previously cloned a novel AT1R-associated protein (ATRAP) that has three transmembrane domains and specifically interacts with the C-terminal cytoplasmic domain of the AT1R. This protein has been reported to modulate AT1R function in COS-7 cells, human embryonic kidney 293 cells and vascular smooth muscle cells [13–15]. In the present study, to clarify the functional importance of ATRAP in cardiomyocytes, we examined whether ATRAP is endogenously expressed in

*Corresponding author. Fax: +81 45 701 3738.
E-mail address: tamukou@med.yokohama-cu.ac.jp (K. Tamura).

Abbreviations: Ang II, angiotensin II; ACE, angiotensin-converting enzyme; AT1R, angiotensin II type 1 receptor; C-terminal, carboxy-terminal; ATRAP, angiotensin II type 1 receptor-associated protein; hemagglutinin epitope-tagged ATRAP, HA-ATRAP; FLAG-tagged mouse AT1R, FLAG-AT1R; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; Ad.HA-ATRAP, adenoviral vector expressing hemagglutinin epitope-tagged ATRAP; Ad.LacZ, adenoviral vector expressing bacterial β-galactosidase; RT, reverse transcriptase; PCR, polymerase chain reaction; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated protein kinase; JNK, c-Jun N-terminal kinase; p38MAPK, p38 mitogen-activated protein kinase; SRE, serum response element; AT2R, angiotensin II type 2 receptor

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cardiomyocytes and analyzed the function of ATRAP in the Ang II-induced hypertrophic responses of cardiomyocytes.

2. Materials and methods

2.1. Cell culture

The immortalized cardiomyocyte cell line H9c2, which expresses endogenous AT1R, was cultured as described previously [16].

The primary culture of neonatal mouse cardiomyocytes was prepared using the method originally described by Goshima [17] with minor modifications [18,19].

2.2. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of ATRAP

Total RNA from cardiomyocytes was isolated by the single-step guanidinium thiocyanate–phenol–chloroform method using ISOGEN (Nippon Gene). One microgram of total RNA was reverse-transcribed into cDNA with 1 U/ml reverse transcriptase (Superscript, Invitrogen) at 37 °C for 1 h in standard buffer. For the amplification of ATRAP cDNA, the following oligonucleotide primers were designed [from nucleotide +90 to +333 (a 244-bp fragment)]: sense primer 5′-TGCTTGGGGCAACTTCACTATC-3′; antisense primer 5′-ACGGTGCATGTGGTAGACGAG-3′ [13]. The reaction was carried out in a standard reaction mixture and PCR products were analyzed on a 1.6% agarose gel.

2.3. Production of rabbit anti-ATRAP antibody

A 14-aa synthetic peptide corresponding to amino acids 436–449 of the C-terminal tail of the mouse ATRAP was produced using standard solid-phase peptide synthesis techniques. Analysis using the BLAST computer program showed no significant overlap of the immunizing peptide with any known eukaryotic protein. The peptide was purified, conjugated and injected three times intradermally into rabbits at two-week intervals for production of polyclonal antiserum. The rabbits developed ELISA titers >1:128 000 prior to exsanguination. Anti-ATRAP polyclonal antibodies were affinity-purified and used in the present study.

2.4. Western blot analysis of ATRAP

Whole cellular extracts from cardiomyocytes were separated on a 12% SDS–polyacrylamide gel (PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane. The membrane was immunoblotted with anti-ATRAP polyclonal antibody or anti-AT1R polyclonal antibody (Santa Cruz) using the ECL System (Amersham).

2.5. Transient transfection and co-immunoprecipitation

The NH2-terminal hemagglutinin epitope-tagged ATRAP (HA-ATRAP) [14] in pcDNA3 was transiently co-transfected with a FLAG-tagged mouse AT1R (FLAG-AT1R) in pcDNA3.1 [11] in cardiac H9c2 cells according to the PolyFECT protocol (QIAGEN). The ratio of AT1R to ATRAP DNA was 1:3. Forty-eight hours after the transfection, the cells were treated with 100 nM Ang II for 60 min, and crude membrane fractions prepared from the transfected cells [20] were solubilized in 50 mM Tris–HCl (pH 7.5), 140 mM NaCl, 1 mM $CaCl_2$, 1 mM phenylmethylsulfonyl fluoride and 1 mg of aprotinin/ml (buffer A) in the presence of 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid. The mixture was gently agitated for 30 min at 4 °C and thereafter centrifuged at 13 000*g* for 20 min. Cleared supernatants (100 μg of protein) which contain the plasma membrane and endosome fractions [21] were diluted 1:10 in buffer A and incubated for 2 h at 4 °C with anti-FLAG M1 monoclonal antibody (Sigma) and protein G Sepharose (Amersham Biosciences). The beads were then washed in buffer A and the samples were subjected to sodium SDS–PAGE, transferred to PVDF membrane and probed with an anti-HA polyclonal antibody (BETHYL Laboratories, Inc.).

2.6. Immunofluorescence

Cardiac H9c2 cells or neonatal mouse cardiomyocytes were co-transfected with HA-ATRAP and FLAG-AT1R using the method described above. Forty-eight hours after the transfection, the cells were treated with 100 nM Ang II for 60 min and then incubated in fresh medium for 60 min to remove Ang II. The cells were then fixed and permeabilized with 2% paraformaldehyde and 0.1% Triton X-100, respectively. HA-ATRAP was detected with rabbit anti-HA antibody and CY3-labeled anti-rabbit IgG (red label) (Amersham Pharmacia Biotech). AT1R was detected with mouse anti-FLAG M1 antibody and Alexa Fluor 488-labeled anti-mouse IgG (green label) (Molecular Probes), as the secondary antibody. For visualizing Golgi apparatus and endosomes, we used the Golgi marker vector (BD Biosciences Clontech) and the endosome marker (BD Biosciences Clontech) [14].

2.7. Preparation of recombinant adenoviral vectors and gene transfer

Adenoviral vectors were prepared using cDNAs coding for HA-ATRAP (Ad.HA-ATRAP) and bacterial β-galactosidase (Ad.LacZ) using a commercially available system (Adeno X Expression System, Clontech). The virus titer was determined with a plaque assay.

The Ad.HA-ATRAP or the Ad.LacZ (5×10^9 pfu/ml) was transfected into cells. All experiments were performed 48 h after infection.

2.8. Cell surface AT1R binding assay

Cardiomyocytes were seeded in 24-well plates the day before the gene transfer. The Ad.HA-ATRAP or the Ad.LacZ (5×10^9 pfu/ml) was transfected into cardiac myocytes. Forty-eight hours after the transfection, the cells were treated with 100 nM Ang II for 60 min and then incubated in fresh medium for 60 min to remove Ang II. AT1R binding was measured as described previously [22]. Briefly, after two washes with phosphate-buffered saline (PBS) containing 0.1% BSA, the cells were incubated for 1 h at 37 °C with 0.2 nM ^{125}I-[Sar1,-Ile8]Ang II in the absence (for the total count) or presence of 1 mM CV11974. The cells were then washed twice with ice-cold PBS containing 0.1% BSA and were lysed in 0.5 N NaOH. The radioactivity of the lysate was measured with a gamma counter. AT1R binding was calculated as the difference between the total count and the count from samples incubated with CV11974.

2.9. Determination of mitogen-activated protein kinase (MAPK) activity

Transfected cardiomyocytes were stimulated as indicated. After treatment, the cells were extracted with sample buffer and appropriately diluted to give equal protein amounts and then used for SDS–PAGE. Western blot analysis was performed for extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 MAPK (p38MAPK) using anti-active ERK1/2, JNK and p38MAPK polyclonal antibodies (Promega), which recognize only activated ERK1/2, JNK and p38MAPK that is phosphorylated. To detect ERK1/2 and p38MAPK, anti-ERK1/2 polyclonal antibody (Upstate Biochem) and anti-p38MAPK polyclonal antibody (Promega) were used, respectively. To detect JNKs, a mixture of anti-JNK1 monoclonal antibody (Pharmingen) and anti-JNK2 monoclonal antibody (Santa Cruz) was used. The enzyme activity was detected with the ECL Plus System (Amersham) and luminescence was quantified with a FUJI Las3000 Luminescence Image Analyzer.

2.10. Transcriptional fos promoter assay

Cardiomyocytes were seeded in 24-well plates and transfected with the c-fos luciferase reporter gene (p2FTL, 1 μg) using SureFECTOR (B-Bridge International Inc.) according to the manufacturer's instructions. This gene consists of 2 copies of the *c-fos* 5′-regulated enhancer element (−357 to −276) containing a serum response element (SRE), the herpes simplex virus thymidine kinase gene promoter (−200–70), and the luciferase gene [23]. To normalize the transfection efficiency, we employed a dual reporter assay system, in which the pRL-SV40 plasmid (Promega), containing the sea pansy luciferase gene under the control of the SV40 early enhancer/promoter, was co-transfected as an internal control. The transfected cells were incubated with serum-free medium for 48 h. Then the cells were treated with Ang II (100 nM) for 4 h, washed with PBS, and lysed for 15 min with 100 μl of cell lysis buffer (Promega Corp) at room temperature. Finally, 20 μl of the cell extract was mixed with 100 μl of a luciferase assay reagent (Promega Corp), and luciferase activity was measured as described previously [24].

2.11. Incorporation of [^{3}H]phenylalanine

Cardiomyocytes were incubated with 4 μCi/ml of [^{3}H]phenylalanine in the culture medium, which contained 0.36 mM unlabeled phenylalanine, then stimulated with Ang II for 24 h. Cells were rinsed three times with cold PBS and treated with 1 ml of 10% (w/v) trichloroacetic acid for 60 min at 4 °C to precipitate proteins. The precipitate was washed three times with 95% (v/v) ethanol and resuspended in 0.2 M NaOH. Aliquots were measured in a liquid-scintillation counter [25].

2.12. Statistical analysis

Values are expressed as means ± SE in the text and figures. The data were analyzed using ANOVA. If a statistically significant effect was found, a post hoc analysis was performed to detect the difference between the groups. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Cardiomyocytes express ATRAP mRNA and protein

Mouse heart and primary cultured mouse cardiomyocytes express ATRAP mRNA as detected by RT-PCR (Fig. 1A). To demonstrate the endogenous expression of ATRAP protein in cardiomyocytes, we generated affinity-purified polyclonal rabbit antibodies directed at the C-terminal region of the molecule. Western blot analysis revealed ATRAP protein expression in the mouse heart and cardiomyocytes (Fig. 1B). The identity of the bands was confirmed from the apparent molecular weight and co-migration with the signal from the in vitro transcribed and translated ATRAP protein. The reaction mixture from a control empty vector did not exhibit a similar signal (data not shown).

To verify the intracellular distribution of endogenous ATRAP in cardiomyocytes, an immunocytochemical analysis was performed. Immunofluorescence staining of cardiomyocytes revealed a specific particulate localization of endogenous ATRAP protein in the perinuclear region of the cell (Fig. 1C).

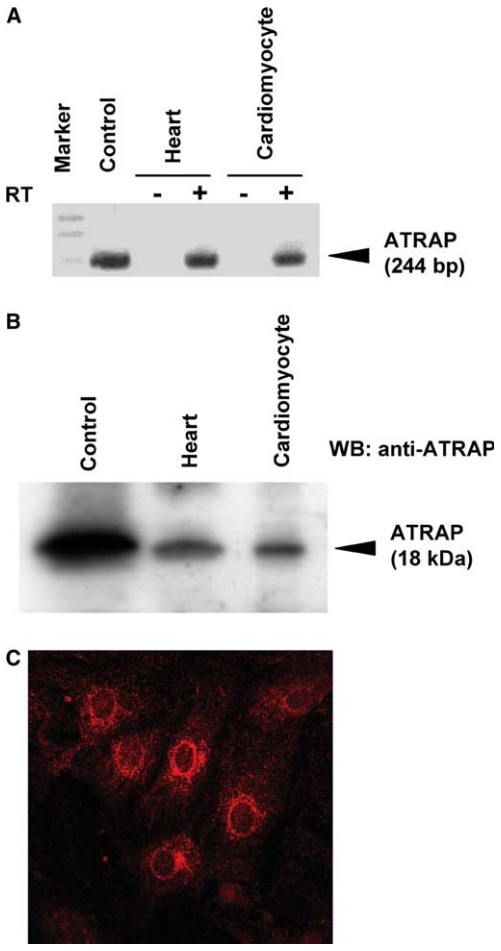

Fig. 1. Demonstration of the endogenous expression of ATRAP at the mRNA and protein level in cardiomyocytes. (A) RT-PCR analysis of endogenous ATRAP mRNA in total RNA from mouse heart and cardiomyocytes. RT-PCR products were visualized by ethidium bromide staining. A DNA marker (Marker), the positive control using ATRAP cDNA plasmid (Control), and the negative control consisting of RT-PCRs lacking reverse transcriptase (RT−) are also shown. (B) Western blot analysis of endogenous ATRAP protein with anti-ATRAP polyclonal antibody in whole cellular extracts from mouse heart and cardiomyocytes. The positive control, using in vitro transcribed/translated ATRAP protein (Control), is also shown. (C) Subcellular distribution of endogenous ATRAP in cardiomyocytes based on immunofluorescent detection. Cardiomyocytes were fixed and co-incubated with rabbit anti-ATRAP antibody. Endogenous ATRAP was detected using CY3-labeled anti-rabbit IgG (red label) as the secondary antibody.

3.2. ATRAP specifically interacts with AT1R in cardiac H9c2 cells

To confirm the binding of ATRAP to the full-length AT1R in cardiac H9c2 cells, a co-immunoprecipitation analysis was performed. For the immunodetection of ATRAP, the protein was HA-tagged at the amino terminus, and a polypeptide of expected size was observed by immunoblotting in transfected cells (Fig. 2A, 1st lane). ATRAP was co-immunoprecipitated specifically from the lysates in association with the AT1R (Fig. 2A, 4th lane). ATRAP was not detected in control immunoprecipitates, including those prepared from the cells expressing ATRAP without FLAG-tagged receptors (Fig. 2A, 2nd lane); this result confirmed the specificity of this association in cardiomyocytes.

The co-immunoprecipitation of ATRAP and the AT1R from cell homogenates suggests co-localization of the two proteins in the form of a complex in intact cardiac H9c2 cells. The association of ATRAP with AT1R was further confirmed by immunofluorescence co-localization analysis. In the cells co-transfected with the FLAG-AT1R and HA-ATRAP fusion protein and treated with 100 nM Ang II for 0 and 60 min, the AT1R was located at the plasma membrane and in the cytosolic region at time 0 (Fig. 2B, a) and internalized into the perinuclear region at 60 min (Fig. 2B, d). ATRAP was located mainly in the perinuclear cytosolic region at time 0 (Fig. 2B, b) and was still detected in the perinuclear cytosolic region at 60 min (Fig. 2B, e). Superimposition of the images reveals a partial co-localization of FLAG-AT1R and HA-ATRAP in intracellular compartments in unstimulated H9c2 cells (Fig. 2B, c), and a substantial Ang II-induced co-localization of the two proteins in intracellular compartments in stimulated H9c2 cells (Fig. 2B, f). Further analysis to determine these compartments showed that HA-ATRAP co-localizes with the Golgi marker ECFP-β-1,4-galactosyl transferase and the vesicle marker of the endocytic pathway GFP-rhoB (data not shown).

3.3. Adenoviral transfer of recombinant ATRAP decreases AT1R expression on the cell surface in cardiomyocytes

To elucidate whether ATRAP is involved in the regulation of AT1R function in cardiomyocytes, we used an

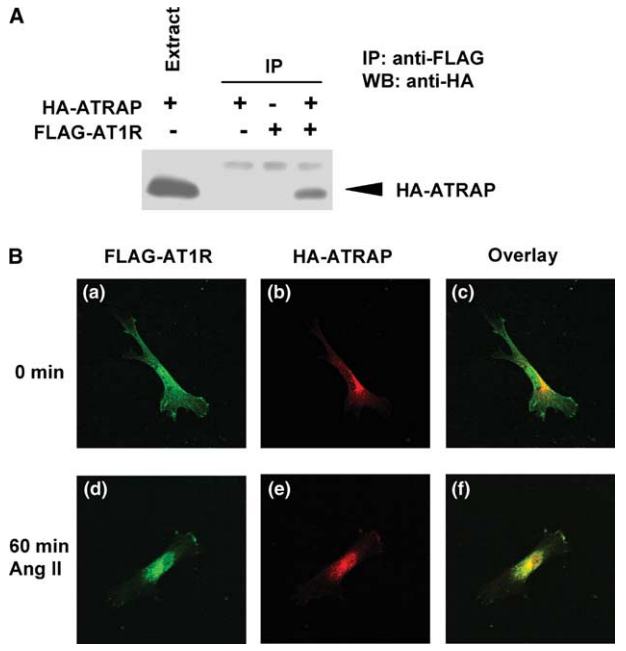

Fig. 2. Demonstration of specific binding and co-localization of ATRAP and AT1R. A cardiac H9c2 cells were transiently co-transfected with HA-tagged ATRAP and FLAG-tagged AT1R. (A) The plasma membrane and endosome fractions of the cells were prepared, and receptor complexes were immunoprecipitated by anti-FLAG antibody. Immunoprecipitates were subjected to SDS–PAGE, blotted, and probed for the presence of the HA epitope. The positive control using whole cellular extracts (Extract) is also shown. (B) H9c2 cells were fixed under baseline conditions (a–c) or on stimulation with 100 nM Ang II for 60 min (d–f). Localization of FLAG-AT1R was determined by using anti-FLAG antibody and green fluorescence secondary antibody and Localization of HA-ATRAP was detected by using anti-HA antibody and red fluorescence secondary antibody.

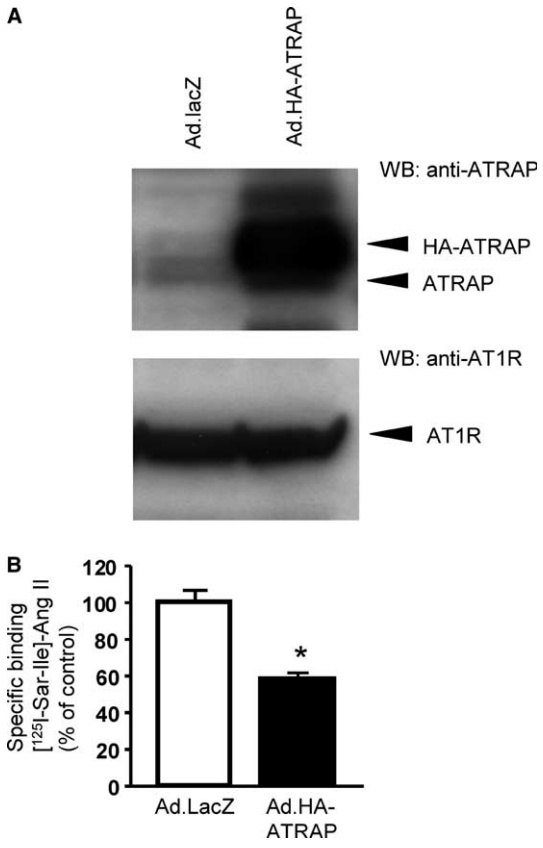

Fig. 3. Expression of HA-ATRAP in cardiomyocytes infected with recombinant adenoviral vectors. (A) Western blot analysis of HA-ATRAP and AT1R with anti-ATRAP polyclonal antibody and with anti-AT1R polyclonal antibody in whole cellular extracts from cardiomyocytes infected with the HA-tagged ATRAP adenoviral vector (Ad.HA-ATRAP) or the LacZ adenoviral vector (Ad.LacZ). The control, using whole cellular extracts from uninfected cardiomyocytes (Control), is also shown. (B) Cell surface AT1R binding assay performed on infected cardiomyocytes as described in Section 2. $^{*}P < 0.05$ versus Ad.LacZ, $n = 4$.

Ad.HA-ATRAP. The results of Western blot analysis using whole cellular extract confirmed that ATRAP was over-expressed in cardiomyocytes, which were infected with Ad.HA-ATRAP (Fig. 3A).

To investigate the possible functional role of a direct interaction between ATRAP and the AT1R, changes in the expression of AT1R were examined by Western blot analysis using whole cellular extracts of cardiomyocytes. Adenoviral transfer of recombinant ATRAP resulted in no apparent changes in the AT1R protein levels in whole cellular extracts from cardiomyocytes (Fig. 3A). Next, to determine the changes in AT1R number on the plasma membrane, cell surface AT1R binding assay in cardiomyocytes was performed. AT1R-ligand binding on the surface of unstimulated cardiomyocytes was downregulated by the adenoviral transfer of recombinant ATRAP (Fig. 3B). Scatchard analysis indicated that the decrease in radioligand binding was due to a change in receptor number on the cardiomyocyte's surface (data not shown).

To further elucidate the possible effect of ATRAP on the internalization of the AT1R, cardiomyocytes infected with Ad.HA-ATRAP were exposed to 100 nM Ang II for 60 min and then incubated in fresh medium for 60 min to remove Ang II. In control cardiomyocytes infected with Ad.LacZ, the AT1R was localized to the plasma membrane under baseline conditions, and stimulation with Ang II for 60 min provoked a significant internalization of the receptor into the perinuclear cytoplasm as detected by immunofluorescence analysis, which effect was followed by a recycling of the receptor to the plasma membrane after removal of Ang II from the medium (Fig. 4A, a–c). On the other hand, cardiomyocytes infected with Ad.HA-ATRAP showed a decrease in the number of AT1R at the plasma membrane and an increase in internalized perinuclear AT1R even under baseline conditions, and the receptor was still localized mainly in the perinuclear region 60 min after removal of Ang II from the medium (Fig. 4A, d–f). Furthermore, the results of the radioligand binding assay also showed that the overexpression of ATRAP significantly inhibited the recovery of cell surface AT1R density induced the by removal of Ang II for 60 min (Fig. 4B).

3.4. ATRAP specifically inhibits Ang II-induced phosphorylation of p38MAPK, transcriptional activity of c-fos, and protein synthesis in cardiomyocytes

We next examined the possible effect of ATRAP on the downstream effectors of the AT1R signaling pathway in cardiomyocytes by performing adenoviral transfer of recombinant ATRAP. Treatment with Ang II significantly augmented the phosphorylation of ERK1/2, JNK, and p38MAPK in cardiomyocytes infected with Ad.LacZ (Fig. 5A–C). Although the Ang II-induced phosphorylation of ERK1/2 and JNK was

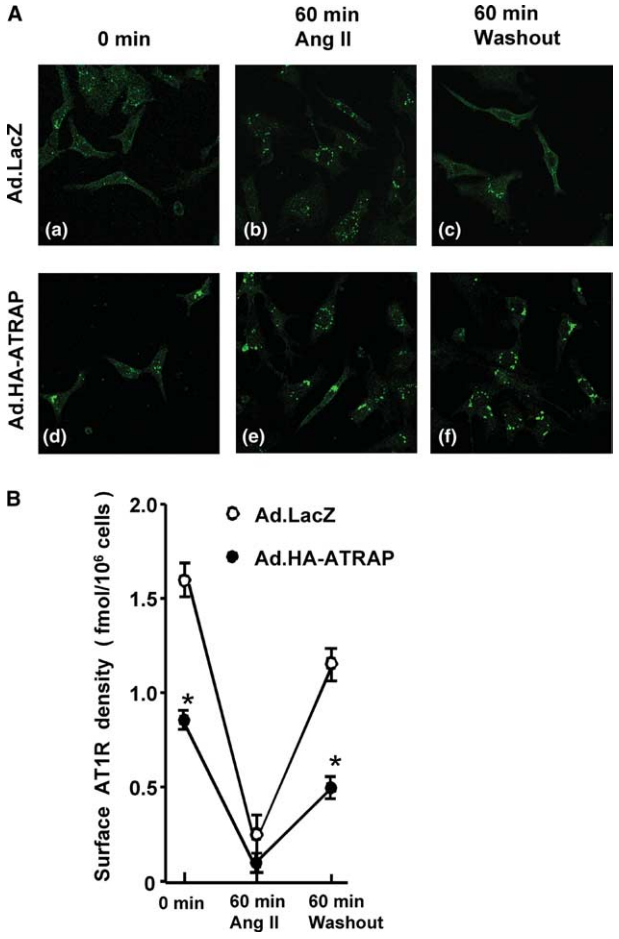

Fig. 4. Promotion of AT1R internalization and inhibition of AT1R recycling to the plasma membrane by overexpression of HA-ATRAP in cardiomyocytes. (A) Cardiomyocytes, which were infected with the HA-tagged ATRAP adenoviral vector (Ad.HA-ATRAP) or the LacZ adenoviral vector (Ad.LacZ), were transiently co-transfected with FLAG-tagged AT1R. Neonatal mouse cardiomyocytes were fixed under baseline conditions (a,d), on stimulation with 100 nM Ang II for 60 min (b,e), or after Ang II removal for 60 min (c,f). Localization of FLAG-AT1R was determined by using anti-FLAG antibody and green fluorescence secondary antibody. (B) Cell surface AT1R binding assay performed on infected cardiomyocytes under baseline conditions (a,d), on stimulation with 100 nM Ang II for 60 min (b,e), and after Ang II removal for 60 min (c,f) as described in Section 2. $^{*}P < 0.05$ versus Ad.LacZ, $n = 4$.

not affected by infection with Ad.HA-ATRAP, the activation of p38MAPK was significantly suppressed by overexpression of HA-ATRAP.

Exposure to Ang II resulted in a significant increase in transcriptional activity of the c-fos promoter luciferase reporter gene in cardiomyocytes infected with Ad.LacZ (Fig. 6A). However, overexpression of HA-ATRAP prevented the increase in activity of the c-fos promoter by Ang II. Protein synthesis was evaluated through the incorporation of [^{3}H]phenylalanine into cardiomyocytes (Fig. 6B). Treatment with Ang II increased the incorporation of [^{3}H]phenylalanine into cardiomyocytes transfected with adenovirus expressing LacZ. Interestingly, infection with Ad.HA-ATRAP significantly inhibited the Ang II-induced stimulation of [^{3}H]phenylalanine incorporation in cardiomyocytes.

4. Discussion

This study reports the following findings. First, evidence is provided for the endogenous expression of ATRAP in cardiomyocytes and its perinuclear localization. Second, ATRAP was found to promote downregulation of the AT1R in cardiomyocytes. Third, ATRAP selectively attenuated Ang II-induced phosphorylation of p38MAPK but not that of ERK1/2 or JNK. Fourth, ATRAP decreased Ang II-induced c-fos promoter transcription and protein synthesis in cardiomyocytes. These results suggest that ATRAP acts as a negative regulator of hypertrophic responses in cardiomyocytes.

There is evidence that mechanical stress, growth factors, cytokines, and vasoactive agents induce hypertrophic responses in cardiomyocytes [26,27]. Although the molecular mechanisms responsible for cardiomyocyte hypertrophy remain to be fully determined, Ang II is reported to be a major contributor to this process [1]. We have previously reported the molecular cloning of ATRAP, a novel AT1R C-terminal-interacting molecule, and the expression of ATRAP mRNA in the heart [13]. However, it had not been previously clarified whether ATRAP was actually expressed in cardiomyocytes and whether it was functionally linked to AT1R signaling (in cardiomyocytes).

A previous study by our laboratory showed that ATRAP contains three hydrophobic domains at the amino-terminal end of the protein and a hydrophilic cytoplasmic carboxyl-terminal tail, indicating that ATRAP is a transmembrane protein [14]. Subcellular localization is critical for the action and regulation of the AT1R [12]. Thus, an important way to explore cardiac ATRAP function is to determine whether ATRAP localizes to cellular areas compatible with receptor activity. In our previous studies using non-cardiac cell lines, ATRAP was found to be localized both at the plasma membrane and in intracellular vesicular compartments corresponding to the endoplasmic reticulum, Golgi, and endocytic vesicles on immunofluorescence staining [13,14]. However, the results of the immunocytochemical analysis in the present study showed that endogenous and transfected ATRAP is mainly localized to the perinuclear region in cardiomyocytes, in spite of the finding of some degree of localization of AT1R both at the cell periphery and in the perinuclear cytosole. Interestingly, in contrast to our previous observation of a considerable co-localization of ATRAP and AT1R at the periphery and in intracellular compartments in non-cardiac cells [13,14], Ang II significantly facilitated co-localization of the two proteins in a perinuclear region but not at the cell periphery in cardiomyocytes, as was revealed on immunofluorescence staining. These results suggest that the association of ATRAP with the AT1R mainly occurs in perinuclear vesicular membranes such as the Golgi, and also in endocytic vesicles in cardiomyocytes, and that ATRAP does not directly interact with the AT1R at the cell surface. The results of the radioligand binding assay showed that the number of AT1R at the cell surface decreased with the overexpression of ATRAP, which also supports the notion that ATRAP promotes the downregulation of the AT1R.

Hypertrophic responses to Ang II are mediated directly by several intracellular signaling cascades through activation of the AT1R [28]. Because the MAPK family is proposed to play a critical role in Ang II-induced cardiac hypertrophy and remodeling [29,30], we examined the involvement of the

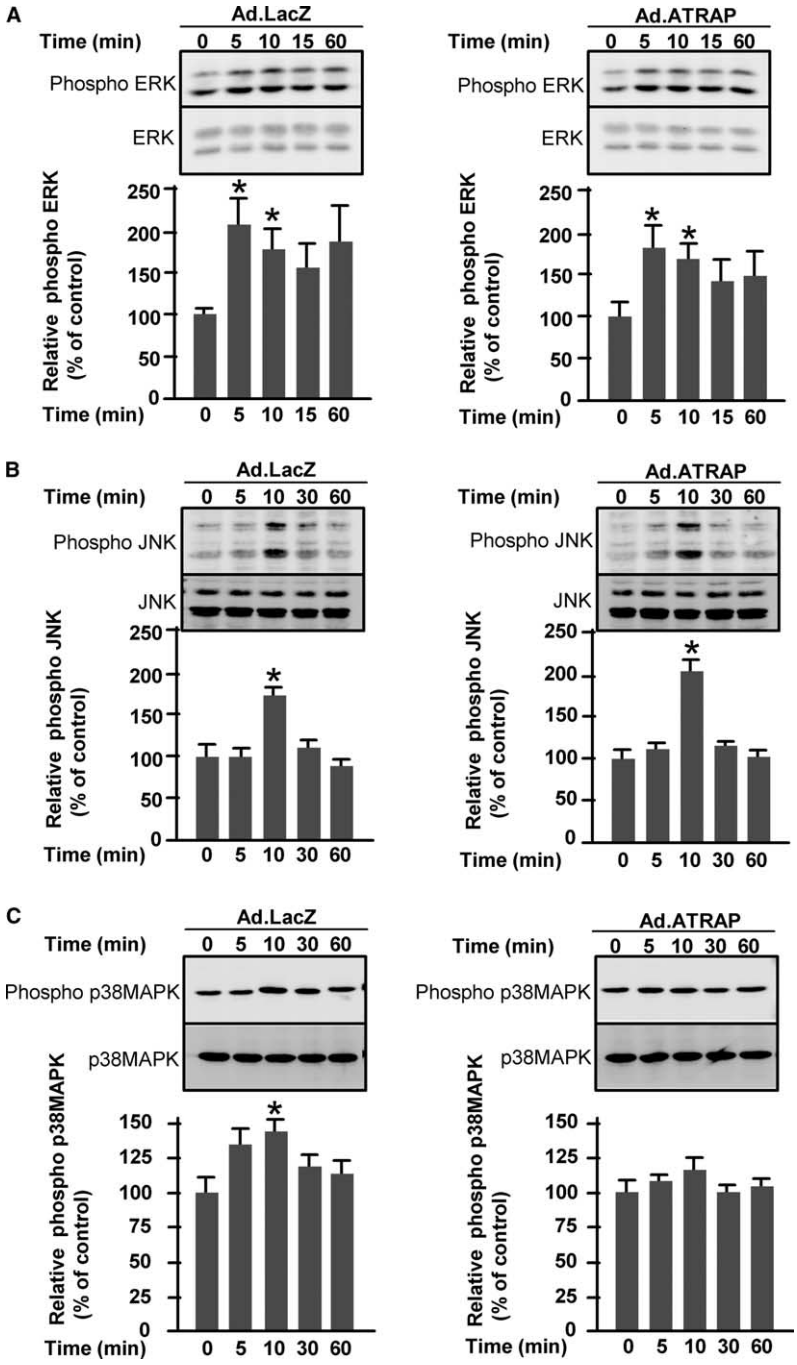

Fig. 5. Inhibitory effect of ATRAP on Ang II-induced activation of p38MAPK in cardiomyocytes. Cardiomyocytes were infected with the HA-tagged ATRAP adenoviral vector (Ad.HA-ATRAP) or the LacZ adenoviral vector (Ad.LacZ), and maintained in serum-free medium for 48 h. Then the infected cells were stimulated with 100 nM Ang II for various periods of time, as indicated. The cell lysates were subjected to immunoblotting for phospho- and total ERK1/2 (A), phospho- and total JNK (B), and phospho- and total p38MAPK (C) as described in Section 2. $^{*}P < 0.05$ versus time 0, $n = 6$.

MAPK family in the effect of ATRAP on cardiomyocytes. Adenovirus-mediated overexpression of ATRAP in cardiomyocytes was accompanied by less Ang II-induced activation of p38MAPK than in those cells infected with Ad.LacZ. On the other hand, Ang II-induced phosphorylation of neither ERK1/2 nor JNK was inhibited by the overexpression of ATRAP. Earlier, we had speculated that ATRAP broadly attenuates AT1 receptor-mediated signaling [15]. However, the present results suggest that the interaction between ATRAP and the AT1R selectively suppressed the p38MAPK pathway. This raises the possibility that internalization inhibits mainly a non-Gq-mediated pathway [31]. The hypertrophic effect of the MAPK family on cardiomyocytes has not yet been clarified in detail, and conflicting findings have been reported on whether activation of ERK1/2 or JNK mediates the specific changes in gene expression associated with the pathologic hypertrophic phenotype [32–34]. However, there is evidence which has been reported which suggests that p38MAPK is able

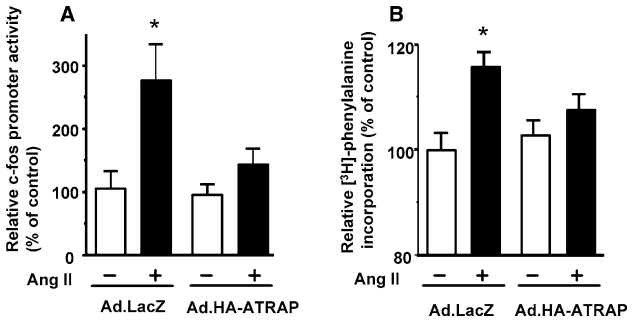

Fig. 6. Inhibitory effect of ATRAP on Ang II-induced hypertrophic responses in cardiomyocytes. (A) Transcriptional fos-luciferase assay. Cardiomyocytes were infected with the HA-tagged ATRAP adenoviral vector (Ad.HA-ATRAP) or the LacZ adenoviral vector (Ad.LacZ), transiently co-transfected with the c-fos promoter luciferase gene, and treated with 100 nM Ang II for 4 h. The transcriptional activity of the c-fos promoter was assessed as described in Section 2. $^{*}P < 0.05$ versus control, $n = 5$. (B) [^{3}H]phenylalanine incorporation assay. Mouse cardiomyocytes were infected with the Ad.HA-ATRAP or the Ad.LacZ, and treated with 100 nM Ang II for 24 h. [^{3}H]phenylalanine incorporation was determined as described in Section 2. $^{*}P < 0.05$ versus control, $n = 8$.

to mediate the development of pathologic cardiomyocyte hypertrophy [31,33,35–37].

Consistent with the role of p38MAPK activation in the hypertrophic growth program of cardiomyocytes through SRE-mediated transcriptional stimulation [35], we observed a significant inhibition of Ang II-stimulated transcriptional activation of the c-fos promoter, which contains SRE, as well as Ang II-induced increase in protein synthesis. Taken together, these results suggest that ATRAP may suppress the AT1R-mediated cardiomyocyte hypertrophy through an inhibition of the p38MAPK pathway.

In conclusion, this study provides new and important information on the functional significance of ATRAP as a negative regulator in cardiomyocyte hypertrophy. These results suggest that the activation of ATRAP could be useful to suppress cardiomyocyte hypertrophy. However, further work is needed to obtain direct *in vivo* evidence of the effects of ATRAP on inhibition of cardiac hypertrophy, as well as the specific mechanisms by which it exerts this effect. Specific experiments for this purpose are currently in the planning stage and are expected to be undertaken at the earliest opportunity.

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