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

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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

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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].