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Original article

Nuclear accumulation of the AT_1 receptor in a rat vascular smooth muscle cell line: effects upon signal transduction and cellular proliferation

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Abstract

The objective of the study was to identify the functional outcome of intracellular versus extracellular angiotensin II–AT_1 receptor interactions in vascular cells. Rat vascular smooth muscle cell line A10 was transfected, independently and concurrently, with plasmids encoding fluorescent fusion proteins of rat angiotensin II (*pECFP/AII*, encodes AII fused downstream of enhanced cyan fluorescent protein) and the rat AT_{1a} receptor (*pAT1R/EYFP*, encodes the rat AT_{1a} receptor fused upstream of enhanced yellow fluorescent protein). The AII fluorescent fusion protein possesses no secretory signal peptide and deconvolution microscopy established that is maintained within these cells predominantly in the nucleus. AT_1R/EYFP was absent from the nucleus when expressed exclusively or in untreated cells but accumulated in the nucleus following exogenous AII treatment or when co-expressed with ECFP/AII. Furthermore, expression of ECFP/AII stimulated proliferation of A10 vascular smooth muscle cells (VSMCs) 1.6-fold ($P < 0.05$). Transfection of a control, *pECFP/AII$_C$* (which encodes a scrambled AII peptide fused to ECFP) had no growth effect. In light of the intracellular growth effects of ECFP/AII, we sought to elucidate the underlying signaling pathways. We found that *extracellular* AII treatment of A10 cells activated cAMP response element-binding protein (CREB) as determined by one-hybrid assays and immunoblots. Expression of *intracellular* ECFP/AII similarly activated CREB. However, intracellular and extracellular AII activated CREB through different phosphorylation pathways. Exogenous AII treatment of A10 cells activated p38MAPK and ERK1/2 phosphorylation as determined by Western blot analyses and one-hybrid assays. The p38MAPK inhibitor, SB203580, and the ERK kinase inhibitor, PD98059 each partially inhibited exogenous AII-conferred CREB activation confirming that p38MAPK and ERK1/2 mediate CREB phosphorylation in this system. In contrast, expression of ECFP/AII (intracellular AII) in A10 VSMCs activated p38MAPK but not ERK1/2; inhibition of p38MAPK by SB203580 inhibited intracellular AII-induced CREB phosphorylation. In summary, extracellular AII stimulates at least one pathway common to intracellular AII. This common pathway, in the case of exogenous AII, likely reflects intracellular signaling following internalization of receptor–ligand complex. Extracellular AII *also* stimulates a unique pathway, apparently reflecting interaction with plasma membrane-associated AT_1R.
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1. Introduction

We have reported that co-expression of a fluorescent fusion protein of AII (ECFP/AII) with a fluorescent fusion protein of the AT_1 receptor (AT_1R/EYFP) alters the receptor distribution and increases proliferation in COS-7 and CHO-K1 cells [1]. The present studies were designed to confirm these physiological effects in vascular smooth muscle cells (VSMCs) and to identify downstream signaling pathways affected by expression of intracellular AII (ECFP/AII). Since exogenous AII has been reported to stimulate CREB phosphorylation in several systems, we investigated the potential for intracellular AII–AT_1 receptor interactions to stimulate CREB activation in A10 VSMCs (which express the AT_1R [2–5]). We further investigated the kinase pathways involved in CREB phosphorylation activation by intracellular AII (IC AII). Cammarota et al. [6] report that extracellular AII stimulates CREB phosphorylation in bovine adrenal chromaffin cells through an ERK1/2-depen-

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dent mechanism. In VSMCs, however, exogenous AII induces activation of CREB and transcription from the fibronectin promoter via p38MAPK activation [7]. Furthermore, studies by Ichiki and associates (Funakoshi et al. [8]) suggest that p38MAPK, ERK1/2 and PKA may all be involved in AII-mediated CREB phosphorylation and downstream *c-fos* expression, protein synthesis and VSMC hypertrophy. Based on these principles, we sought to determine whether intracellular AII similarly activates multiple signaling pathways in VSMCs and to what extent these overlap with pathways activated by extracellular AII.

2. Materials and methods

2.1. Plasmids

pECFP-C1 and *pEYFP-N1* (otherwise referred to as *pECFP* and *pEYFP* in this paper) are control vehicles into which desired fusion protein-encoding DNA sequences can be cloned (Clontech, Palo Alto, CA, USA). In the C1 vector, encoded fused proteins are present at the C-terminus of the fluorescent protein moiety; in the N1 vector, encoded fused proteins are present at the N-terminus. *pAT$_1$R/EYFP* (encodes a fusion protein of the rat AT$_1$R), *pECFP/AII* (encodes a fusion protein of AII) and *pECFP/AII$_C$* (encodes a scrambled AII peptide) have been previously described [1].

2.2. Transfections

Transfection efficiency was compared and optimized using several reagents. Lipofectamine 2000, Lipofectin (Invitrogen Corp., Carlsbad CA, USA) and Fugene 6 (Roche Biochem., Indianapolis IN, USA) were compared for transfection efficiency while varying the amount of control plasmid, *pEYFP-N1* and lipid reagent. Lipofectamine 2000 was found to be slightly superior for these cells; the optimum ratio of DNA-to- lipid reagent and the optimum conditions are described in the section below and apply for all transfections.

2.3. Western blots

The clonal cell line A10 (ATCC CRL 1476) was derived from the thoracic aorta of DB1X embryonic rat and possesses many of the properties characteristic of smooth muscle cells. The cells produce spontaneous action potentials at the stationary phase of the growth cycle and exhibit an increase in activity of the enzymes myokinase and creatine phosphokinase. In addition, these cells have been shown to express both the angiotensin AT$_1$ receptor and a number of contractile proteins characteristic of VSMCs [2–5,9]. Line A10, passage 15–20, was seeded at 2×10^5 cells/35 mm dish in DMEM, 10% FBS. 24 h after plating, cells were transfected overnight using 0.5 μg of each DNA and 1.25 μl of Lipofectamine 2000 (1 μg/μl) (Invitrogen Carlsbad CA) in Optimem low-serum medium. Where indicated, ~48 h post-transfection, cells were rendered quiescent in 0.5% FBS-containing media for 3 h and then treated with SB203580 (10 μmol/l) or PD98059 (30 μmol/l) for 30 min. Cadmium chloride (20 μmol/l) was used as a positive control in some samples and was added during the 3 h low-serum incubation prior to harvest. AII was added for 15 min immediately prior to harvest where indicated (for data presented in Figs. 10–12A). Total cell extract was collected [10] and protein measured. Protein was electrophoresed on a 4–12% NuPAGE Bis-Tris gradient gel and transferred to a Hybond-P–PVDF membrane. Specific immunoreactive proteins were detected using the ECL Plus Western blotting detection reagents together with sheep anti-AT$_1$R (human), rabbit anti-sheep IgG (both from Abcam Inc., Cambridge, MA, USA). Anti-phospho-p38MAPK, anti-p38MAPK, anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-CREB and anti-CREB antibodies were from Cell Signaling Technology Inc. (Beverly, MA, USA). Mouse monoclonal anti-histone antibody and mouse monoclonal alpha-tubulin antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were used as controls for nuclear and cytosolic fractions, respectively. ECFP/AII was identified using rabbit anti-AII (human) antibodies (Peninsula Laboratories, San Carlos CA, USA). EYFP was detected using anti-GFP (FL) antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz CA, USA).

2.4. Subcellular fractionation

Cells were plated at 2×10^6/100 mm dish and then were either transfected with *pAT$_1$R/EYFP* (and treated with AII for 30 min prior to harvest), or transfected with *pECFP/AII*. Extracts were collected 72 h post-transfection and fractionated by a modification of the method of Maloney et al. [11]. Specifically, plates were washed with 4 ml cold PBS and (Step 1) scraped in 500 μl of CSK buffer [10 mM Tris–HCl (pH 6.8), 3 mM $MgCl_2$, 100 mM NaCl, 1 mM EGTA, 300 mM sucrose, 1 mM DTT, 1 mM PMSF, 10 mM NaF, 1 mM $NaVO_3$ and phosphatase inhibitor]. Samples were transferred to a Dounce homogenizer and lysed using 30 strokes of a B-type pestle. Lysis was confirmed by microscopy using trypan blue staining. Lysate was transferred to an Eppendorf and centrifuged for 10 min at $1000 \times g$. (Step 2) The pellet from this step was resuspended in 500 μl of CSK buffer and centrifuged for 5 min at 4 °C, $2000 \times g$. The resulting pellet was then resuspended in 200 μl CSK/1% TX-100 buffer, incubated on ice for 5 min, and then centrifuged at $10{,}000 \times g$ for 5 min at 4 °C. The supernatant was diluted with Laemmli sample buffer and represents the nuclear fraction.

(Step 3) The supernatant from Step 1 was centrifuged at $8000 \times g$ for 10 min at 4 °C. (Step 4) The supernatant was removed and centrifuged in SW50.1 rotor (35,000 rpm for 30 min at 4 °C). The resulting supernatant was diluted with 4X Laemmli sample buffer to 1X for cytoplasmic fraction.

The pellet from Step 4 was resuspended in CSK/1% TX-100 buffer, incubated on ice for 30 min, and then centrifuged at $100{,}000 \times g$ for 30 min. The supernatant was diluted with Laemmli sample buffer (represents membrane fraction). Wes-

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