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Angiotensin AT2 receptor activates the cyclic-AMP signaling pathway in eel

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ABSTRACT

A unique angiotensin type 2 receptor (AT2) that induces a cAMP signaling pathway was cloned and characterized for the first time in fish, *Anguilla japonica*. Phylogeny and synteny results showed that the AT2s among fishes and tetrapods share the same origin despite a sub-cluster formation among eel, salmon, and zebrafish. The eel AT2 was expressed abundantly in the spleen and localized at straight arterioles and ellipsoid regions prior to the sinusoid, suggesting a role in the regulation of microcirculation and/or immune response. Various angiotensin (Ang) peptides, including Ang II, Ang III, and Ang IV, were detected in the spleen by a radioimmunoassay coupled with HPLC separation, and these endogenous peptides stimulated a cAMP signaling, which has no crosstalk with cGMP pathway. The common and contrasting features of AT2 between fishes and mammals imply some ancestral characters of AT2, which are important information for receptor binding and evolutionary studies.

1. Introduction

Angiotensin type 1 and type 2 receptors (AT1 and AT2) belong to the seven transmembrane G-protein coupled receptor (GPCR) superfamily. Many of the classical angiotensin II (Ang II) functions such as vasoconstriction and thirst induction are mediated via AT1 but less is known about the function and properties of AT2 (Fyhrquist and Saijonmaa, 2008). The AT2 may offset or oppose the AT1-mediated actions on cell growth, blood pressure, and fluid intake, and may mediate anti-growth and apoptotic actions (Gallinat et al., 2000). The AT2 displays the hallmark motifs and signature residues of a GPCR, but fails to demonstrate most of the classic features of GPCR such as cAMP or Ca^{2+} signaling (Porrello et al., 2009). The AT2 was often termed as unconventional, enigmatic, and atypical (Steckelings et al., 2005) and countless effort have been made to consolidate its functions.

Involvement of G_i-protein was postulated for some AT2-mediated functions in mammals as the effects are sensitive to pertussis toxin (Gallinat et al., 2000; Kang et al., 1994). Three specific phosphatases were identified to be stimulated upon AT2 activation: mitogen-activated protein kinase phosphatase 1 (MAPK-1) (Horiuchi et al., 1997), SH2 domain-containing phosphatase 1 (SHP-1) (Bedecs et al., 1997), and protein phosphatase 2A (PP2A) (Huang et al., 1995). The AT2 also modulated the cGMP/nitric oxide pathway via both soluble and particulate guanylyl cyclases (GC), but the increase or decrease in cGMP was highly dependent on the cell types used in the experiments (Gallinat et al., 2000; Steckelings et al., 2005). The recent inclusion of AT2 receptor interacting protein (ATIP) has added complexity to the AT2 signaling and regulation (Rodrigues-Ferreira and Nahmias, 2010). The AT2 is widely distributed in fetal tissue but its expression is dramatically decreased after birth, being restricted to a few organs such as brain, adrenal, heart, vascular endothelium, kidney, myometrium and ovary (de Gasparo et al., 2000).

Studies in non-mammalian AT2 were highly limited. An AT2-like receptor was suggested in the endothelium of bird abdominal aorta by inhibitor studies (Nishimura, 2001). AT1 and AT2 were studied in fishes using commercial pharmacological drugs and analogues, but most of the results were negative or inconsistent (Russell et al., 2001). Such failure to produce reliable results could be due to the lack of specificity of these drugs to fish receptors or the intrinsic differences of the receptor characteristics. Neither by cloning nor by inhibitor analysis has AT2 been characterized in fishes; AT2 receptor antagonist, PD123319, failed to show any bindings or physiological effects (Nishimura, 2001). Increasing evidence showed that the renin angiotensin system (RAS) is involved in ion and osmotic regulation in fishes, in particular in

Abbreviations: ATIP, AT2 receptor interacting protein; CRE, cAMP-responsive element; DIG, digoxigenin; GC, guanylyl cyclase; GPCR, G-protein coupled receptor; HEK293, human embryonic kidney cell line 293; KT5823, 2,3,9,10,11,12-hexahydro-10R-methoxy-2,9-dimethyl-1-oxo-9S,12R-epoxy-1H-diindolo[1,2,3-fg:3′,2′,1′-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid, methyl ester; MS-222, ethyl 3-aminobenzoate methanesulfonate; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; PKA, protein kinase A; PKC, protein kinase C; PP2A, protein phosphatase 2; PTPase, protein tyrosine phosphatase; RAS, renin angiotensin system; SEAP, secreted alkaline phosphatase; SHP-1, SH2 domain-containing phosphatase 1.

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dehydrating conditions such as seawater acclimation (Wong et al., 2006). We recently identified multiple angiotensin peptides in the eel circulation, which point to the complexity of fish RAS (Wong and Takei, 2012). In the present study, we characterized the AT2 in Japanese eel (*Anguilla japonica*) to provide a comparative model from a fish lineage to broaden our perspectives on the AT2. For this purpose, we cloned the AT2 and localized its transcript in the gill and spleen by RT-PCR and at the cellular level by *in situ* hybridization. Subsequently, the gene was transiently expressed in culture cells and its intracellular pathway and the selectivity to homologous angiotensin peptides were determined. Finally, the similarities and variations of AT2 are discussed between fish and mammals.

2. Material and methods

2.1. Animals

Japanese eel (*A. japonica*; 160–210 g) were obtained from a local eel farm and kept in a recirculating aquarium system in the Atmosphere and Ocean Research Institute, the University of Tokyo. The water was maintained at 18 °C and the eel were exposed to a 14 h:10 h light/dark cycle throughout the experiment. The fish was kept in fresh water and not fed during the experimental period. All animal experiments were approved by the Animal Experiment Committee of the University of Tokyo.

2.2. Cloning of eel AT2

Initially, degenerate primers (F: GCTACYTGGCCATCGTSCATCC; R: AGAARGTCACACAGTGGAAMGGGAAC) were designed according to the conserved regions of other vertebrate AT2s for the amplification of a partial sequence of AT2 in eel. After obtaining the partial sequence, specific primers (F: CTGATGAAGAACATCCTGGGC TTCCTG; R: CATTGAGGAAGGTGAGGAGGTGGAAGG) were designed to clone the full-length receptors using the SMART cDNA Library Construction kit (Clontech Laboratories, Mountain View, CA, USA) according to the manufacturer's protocol. All sequencing procedures were performed using a BigDye Terminator Cycle sequencing kit and an ABI 3130 DNA sequencer (Life Technologies, Grand Island, NY, USA).

Eel was terminally anesthetized by 0.1% ethyl 3-aminobenzoate methanesulfonate (MS-222; Sigma, St. Louis, MO, USA) neutralized with sodium bicarbonate. Various tissues including brain, pituitary, gill, atrium, ventricle, liver, kidney, esophagus, stomach, anterior intestine, posterior intestine, spleen, rete mirabilis, and interrenal were dissected and snap frozen in liquid nitrogen, stored at −80 °C until use. Total RNA samples were obtained from these tissues, treated with DNase I to remove genomic DNA, and reverse transcribed using SuperScript III First-Strand Synthesis for RT-PCR (Life Technologies, Grand Island, NY, USA). Negative controls for reverse transcription were performed using the same RNA templates in the reactions without reverse transcriptase. The PCR was performed using an ABI 9700 thermal cycler (Life Technologies, Grand Island, NY, USA) with Takara ExTaq DNA polymerase reagents (Takara Bio Inc., Shiga, Japan), and PCR products were electrophoresed on 1.2% agarose gels stained by ethidium bromide. The optimal cycle numbers for eel AT2 and β-actin are 33 and 24 respectively.

2.3. Bioinformatics analyses

Genome search was performed to collect most of the available AT2 or putative AT2 from each species under genome project coverage for phylogenetic reconstruction. Full-length amino acid sequences of various species were aligned using Mega version 5, and a phylogenetic tree was constructed using the neighbor joining method (Jones–Taylor–Thornton model; Tamura et al., 2011). A bootstrap test was performed with 1000 replicates to validate the phylogenetic relationships. To further validate the phylogenetic relationship, the same alignment was analyzed by Bayesian method (Ronquist and Huelsenbeck, 2003) to generate an alternative phylogenetic tree for comparison. Synteny analysis was performed among the neighbor orthologous genes of AT2 in human, zebrafish, and stickleback to investigate the extent of homology among these orthologs. Three dimensional structure of eel AT2 was modeled using a protein structure prediction server (PS^2) (Chen et al., 2006).

2.4. Determination of endogenous angiotensin peptides in eel spleen

Eel was anaesthetized as described above and the spleen was dissected. The spleen was immediately boiled in 4 volumes of 1 M acetic acid at 100 °C for 5 min and homogenized. Peptide fraction was extracted by the acetic acetone method described elsewhere (Brown et al., 2005). The various angiotensin peptides were resolved by a HPLC system and quantified by a radioimmunoassay as described previously (Wong and Takei, 2012).

2.5. Cell line culture, receptor expression, and reporter gene assay

Human embryonic kidney cell line (HEK 293) was cultured in DMEM (Hyclone SH30022.01, Thermo Scientific, MA, USA) supplied with 10% fetal bovine serum, penicillin–streptomycin (100 U/mL) in a 37 °C moist incubator equilibrated with 95% O_2 and 5% CO_2. The cells were seeded on 24 wells culture plates (Costar 3526, Corning, NY, USA) and only cells with low passage number were used. All transfections and treatments were performed at least twice to confirm the validity.

Full-length cDNA of AT2 of eel (GenBank accession number: GU726142) was sub-cloned onto pcdna 3.1 Hygro + expression vector (Life Technologies, Grand Island, NY, USA). A Kozak sequence (CCACC) was added prior to the start codon of the receptor to facilitate the expression. The intracellular pathway downstream of the eel AT2 was screened using a Pathway Profiling Kit (Takara Bio Inc., Shiga, Japan). The kit contains 8 different reporters that respond to various intracellular pathway stimulations, including Ca^{2+} and cAMP signalings. From this screening, the cAMP-signaling pathway was found responsive and therefore it was subsequently used for further analyses. The AT2 expression vector and the pCRE-SEAP (cAMP responsive element – secreted alkaline phosphatase reporter plasmid; Takara Bio Inc., Shiga, Japan) were transiently transfected into the HEK 293 cell line of >90% confluence using Lipofectamine 2000 reagents accordingly to the manufacturer's protocols (Life Technologies, Grand Island, NY, USA). The overexpressed cells were treated, in duplicate wells, with various concentrations of [Asn^1, Val^5]-Ang II, [Asp^1, Val^5]-Ang II, [Val^4]-Ang III, [Val^3]-Ang IV, and [Asn^1, Val^5]-Ang (1–7) for 48 h. Forskolin (1 μM) was used as a positive control for the cAMP stimulation. Culture medium was collected and SEAP activity was measured by the Chemiluminescent SEAP Reporter Gene Assay Kit (Roche Applied Science, IN, USA) according the manufacturer's protocol. Co-transfected cells were stimulated with [Asn^1, Val^5]-Ang II (10^{-8} M or 10^{-7} M) along with a cGMP agonist (8-Bromo-cGMP, 2 mM), a NO-sensitive guanylyl cyclase inhibitor (ODQ, 10 μM), or a selective cGMP-dependent protein kinase inhibitor (KT-5823, 1 μM) for 24 h and the SEAP activities in the media were measured.