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Angiotensin II AT_2 receptor localization in cardiovascular tissues by its antibody developed in AT_2 gene-deleted mice

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Abstract

In contrast to well-established physiological roles of the angiotensin II type 1 receptor (AT_1), the significance of the type 2 receptor (AT_2) remains largely unclear. AT_2-knockout (AT_2KO) mice have a phenotype associated with mild hypertension. This implies that AT_2 has a role for the regulation of blood pressure. To gain insight into the mechanism by which AT_2 regulates systemic blood pressure, we have investigated the expression of the AT_2 receptor protein in adult rat cardiovascular tissues, using a newly developed polyclonal anti-AT_2 antiserum that was successfully obtained in the AT_2KO mice by immunizing with a peptide fragment of the receptor protein. In blood vessels, a stronger immunoreactivity was observed in endothelial cells than in the muscular media of resistant arteries. In the thoracic aorta, AT_2 was observed only in muscular media. Abundant AT_2 immunoreactivity was detected in perivascular nerve fibers. In the heart, positive immunostaining for AT_2 was restricted to the coronary blood vessels. These data suggest that AT_2 expressed in the vascular endothelial cells and muscular media in resistant arteries may play a pivotal role in systemic blood pressure regulation. AT_2 was observed for the first time in the perivascular nerve fibers and may also play a role in neuronal blood pressure regulation.

Keywords: Antiserum; Immunohistochemistry; Vascular endothelial cell; Coronary blood vessels; Mesenteric arteries; Perivascular nerve fibers

1. Introduction

Two subtypes of receptors for angiotensin II, termed AT_1 and AT_2, have been identified and cloned [1–3]. Both subtypes belong to the G-protein-coupled seven transmembrane receptor super family [1,3]. By contrast to well-established physiological roles of AT_1, the significance of AT_2 remains largely undefined. Recent in vitro studies showed that AT_2 mediates inhibition of cell proliferation and stimulation of apoptosis in various cultured cells [4–6], at least in part through phosphotyrosine phosphatase activation [7–9]. AT_2-null hemizygous mice generated by us [10] and others [11] show a hypertensive phenotype that indicates its importance for the regulation of blood pressure. However, there exist only a few studies elucidating the expression of AT_2 in cardiovascular tissues [12–14]. Previous in situ hybridization studies have revealed that the AT_2 gene is abundantly expressed in various tissues of fetal and newborn rats, whereas its expression is restricted to the adrenal gland, brain, heart, and uterus of adult rats [15]. In the present study, to gain insight into the mechanism by which AT_2 regulates systemic blood pressure, we have investigated the expression of AT_2 receptor protein in blood vessels, including thoracic aorta, mesenteric arteries, and coronary arteries, and heart of adult rats, using a newly developed polyclonal anti-AT_2 antiserum. To the best of our knowledge, this is the first report of immunohistochemical analysis of an antigen using the specific anti-antigen–antibody that was successfully obtained from the antigen-deficient mice by immunizing with a peptide fragment of the antigen protein. This paper is also the first to report AT_2 localization in perivascular nerve fibers.

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2. Materials and methods

2.1. Materials and animals

The original AT_2-null mutant (*Agtr2*$^{-/y}$) mice were produced by homologous recombination in embryonic stem cells derived from strain 129/Ola [10]. Chimeric males were mated with C57BL/6J females such that the genetic background of the mutants consisted of 129/Ola and C57BL/6J. Male Wistar rats were purchased from Harlan Sprague Dawley (Indianapolis, IN). The intracellular third loop (ITL) peptide of AT_2 containing cysteine at its C-terminus (25 mer, GIRKHLLKTNSYGKNRITRDQVLKC) was synthesized by Research Genetics (Huntsville, AL). Culture media were obtained from the DNA Synthesis and Reagent Supply Core facility in the Vanderbilt University Diabetes Center. All other chemicals were of analytical grade. All animals were maintained in a humidity- and temperature-controlled room on 12 h light/dark cycles. All procedures for handling animals were approved by the Institutional Committee for Animal Care and Use of Vanderbilt University.

2.2. Antiserum production

The intracellular third loop peptide (ITL peptide, 24 mer, GIRKHLLKTNSYGKNRITRDQVLK) of the rat AT_2 was selected as the specific antigen for the immunization. The ITL peptide was synthesized and coupled to keyhole limpet hemocyanin by Research Genetics according to the method of amino and sulfhydryl group coupling with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester. Antiserum to AT_2 ITL peptide was raised in 10- to 15-week-old female AT_2 knockout (AT_2KO) mice. Primary immunization was carried out with Freund's complete adjuvant, and booster injections of antigen at 2-week intervals were with Freund's incomplete adjuvant. The immunized mice were bled 2 weeks after the last boost, and then the sera were obtained. In order to increase specificity, the antisera were preabsorbed with acetone-powdered liver homogenate prepared from the AT_2KO mouse. A total of 1.2 ml diluted serum (1:300) was incubated with acetone-powdered liver homogenate (approximately 300 mg) at 4 °C for 16 h immediately before the immunohistochemistry. To confirm the specificity of the antiserum, antiserum was preabsorbed with the acetone-powdered liver homogenate in the presence of 2 mg antigen peptide at 4 °C for 16 h and then used as a negative control for the immunohistocytochemistry.

2.3. Cell culture

COS-7 cells (ATCC, Manassas, VA) untransfected or stably transfected with plasmid containing the rat AT_2 sequence (AT_2/COS-7) were prepared [16] and cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere. The subconfluent cells were utilized for immunocytochemical study in order to evaluate the validity of the antiserum.

2.4. ELISA for AT_2

Ninety-six well plates for enzyme-linked immunosorbent assay (ELISA, MaxiSorp, Nunc, Roskilde, Denmark) were coated with 2.5 μg antigen peptide for each well and incubated with a blocking buffer (Block Ace, Snow Brand, Tokyo, Japan). After washing the well with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-Tween), 50 μl of antiserum diluted at various concentrations was placed in the wells. The wells were then incubated with 50 μl of 1:1000 diluted biotinylated goat antimouse immunoglobulin antibody, washed with PBS-Tween, and further incubated with 50 μl of 1:1000 diluted streptavidin coupled with horseradish peroxidase (Amersham Pharmacia Biotech, Piscataway, NJ). The wells were soaked in the reaction solution (pH 5.0) containing 50 mM disodium phosphate, 24 mM citric monohydrate, 37 mM *o*-phenylenediamine dihydrochloride, and 0.03% H_2O_2. Then, the absorbance at 490 nm was measured. Competition experiments to confirm the specificity of the ELISA were performed with the anti-AT_2 antiserum, which had been preincubated with an excess amount of the ITL peptide (2 mg/ml). Assay was carried out with duplicate determinations.

2.5. Immunocytochemistry

Both COS-7 cells and AT_2/COS-7 cells grown in the culture dishes were fixed in acetone for 10 min at 4 °C. After washing with PBS, the cells were incubated at 4 °C for 16 h with the primary antibody incubated with or without acetone-powdered liver homogenate. After washing with PBS, the positive signals were visualized using the indirect peroxidase-labeled antibody method.

2.6. Immunohistochemistry

Blood vessels, including thoracic aorta and mesenteric arteries, and heart were obtained from 7-week-old male Wistar rats. Tissue specimens were frozen in liquid nitrogen after embedding in OCT compound (Sakura Finetek USA, Torrance, CA). The frozen sections were fixed in methanol and then incubated with the primary antiserum diluted in PBS containing 1% bovine serum albumin at 4 °C for 16 h. After washing with PBS, the positive signals were visualized using the indirect peroxidase-labeled antibody method.

3. Results

3.1. Characterization of AT_2 antiserum

The specificity of the immunoreactivity and the titer of the anti-AT_2 antiserum was evaluated by ELISA. Pretreatment