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Angiotensin II AT_2 receptors regulate NGF-mediated neurite outgrowth via the NO-cGMP pathway

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ABSTRACT

We investigated whether Angiotensin II type 2 (AT_2) receptor activation was involved in NGF-induced nerve regeneration. NGF-mediated neurite outgrowth in cultured dorsal root ganglia (DRG) cells was significantly inhibited by AT_2 receptor antagonist (PD123,319) treatment. AT_2 receptor knockdown also inhibited NGF-mediated neurite outgrowth. To determine the mechanisms, we analyzed the NO-cGMP pathway. The cGMP analog increased NGF-mediated nerve elongation, which inhibited by PD123,319. Furthermore, soluble guanylate cyclase expression was significantly less in NGF and PD123,319 treatment DRG than in NGF treatment alone. These results suggest that NGF-mediated neurite outgrowth is suppressed by AT_2 receptor signaling via the NO-cGMP-PKG pathway.

1. Introduction

Angiotensin II activates two major types of seven-transmembrane domain G-protein coupled receptors, which are angiotensin II type 1 (AT_1) and angiotensin II type 2 (AT_2) receptors. The function of AT_1 receptor is known as a regulator of the cardiovascular system, oxidative stress, and cell proliferation. Another group of angiotensin II receptors, the AT_2 receptors, is predominately expressed in fetal tissue, with limited expression in normal adult tissue [1]. However, under pathological conditions, such as vascular injury or atherosclerosis, AT_2 receptor expression significantly increases [2,3].

AT_2 receptors have been shown to mediate nerve regeneration. Previous studies in our laboratories demonstrated that phenol-induced activation of AT_2 receptors facilitates reinnervation of mesenteric perivascular calcitonin gene-related peptide (CGRP)-containing nerves in nerve-injured rats [4]. CGRP, a major neurotransmitter in sensory nerves, is expressed in nerves and produced in dorsal root ganglia (DRG) [5,6]. We also showed that administration of nerve growth factor (NGF) resulted in a greater density of CGRP-containing nerve fibers with increased AT_2 receptor expression in nerve-injured rat DRGs [4]. NGF is a member of the neurotrophin family, which is involved in nerve proliferation and elongation [7]. Taken together, these observations strongly suggest that NGF might increase CGRP-positives nerves in concert with AT_2 receptor expression. However, the relationship between NGF and the AT_2 receptor in nerve elongation remains unknown. The present study examined whether NGF-mediated CGRP neurite outgrowth is influenced by AT_2 receptor attenuation. We also focused on NGF expression and the AT_2 receptor signaling cascade. NGF signals take place through tropomyosine-related kinase A, which activates common intracellular signaling intermediates, including Ras, mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K), and the serine-threonine kinase protein kinase B (Akt) [8,9]. The AT_2 receptor also signals by activating Ras and Raf, which leads to increased MAPK activation [10,11]. A previous study showed that AT_2 receptor activation induces nitric oxide (NO) production and cGMP accumulation [12]. Furthermore, NO activates tropomyosine-related kinase A phosphorylation [13]. Therefore, the present study investigated whether AT_2 receptor blockade affects NGF-mediated neurite outgrowth via the NO-cGMP-PKG pathway.

Abbreviations: AKT, protein kinase B; AT_1, angiotensin II type 1; AT_2, angiotensin II type 2; CGRP, calcitonin gene-related peptide; cGMP, cyclic guanosine monophosphate; DMEM, Dulbecco's-modified Eagle's medium; DRG, dorsal root ganglia; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LI, like-immunoreactive; MAPK, mitogen-activated protein kinases; NGF, nerve growth factor; NO, nitric oxide; PBS, phosphate buffered saline; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; PKG, protein kinase G; sGC, soluble guanylate cyclase; siRNA, small interfering RNA; TBS, Tris-buffered saline; TBS-T, Tris-buffered saline containing 0.1% Tween 20.

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2. Materials and methods

2.1. Animals

All animal procedures were performed in accordance with the ARRIVE guideline and were approved by the Animal Care and Use Committee of the Okayama University of Science. According to these guidelines, efforts were made to minimize the number of animals and their discomfort.

Adult male C57BL6J mice (25–30 g, purchased from Shimizu Experimental Animals, Shizuoka, Japan) were housed in the Animal Research Center of Okayama University of Science in a controlled ambient temperature of 22 °C with 50 ± 10% relative humidity and a 12 h light/12 h dark cycle (lights on at 8:00 a.m.). The mice were fed a normal chow diet and water *ad libitum*. All procedures were used in accordance with institutional guidelines for animal research, which are equivalent to the Japanese Government Animal Protection and Management Law and the Japanese Government Notification on Feeding and Safekeeping of Animals.

2.2. Preparation of DRG cultures

DRG neurons were isolated from male mice under sodium pentobarbital (50 mg/kg) anesthesia. The DRG were dissected, isolated, and rapidly placed in collagenase (5 mg/mL for 30 min at 37 °C; Sigma Aldrich, Tokyo, Japan) followed by incubation for 30 min at 37 °C with trypsin (GIBCO Thermo Fisher Scientific Inc., Tokyo, Japan) and 1.25 mg/mL and 2.5 mg/mL collagenase, respectively. The ganglia were suspended in phosphate buffered saline (PBS) and then collected by centrifugation, followed by mechanical disassociation by pipetting with Dulbecco's-modified Eagle's medium (DMEM) (GIBCO Thermo Fisher Scientific Inc.) containing 10% fetal bovine serum (GIBCO Thermo Fisher Scientific Inc.), 100 U/mL penicillin, and 100 μg/mL streptomycin. The resulting cell suspensions were plated onto 22.2 mm^3 plates and maintained for 5 days at 37 °C in a humidified incubator gassed with 5% CO_2 and air.

To analyze neurite outgrowth of DRG cells, the cells were treated in the absence or presence of drugs: NGF (100 ng/mL; Toyobo, Tokyo, Japan); Na-Nicotinoyl-Tyr-(Na-Cbz-Arg)-Lys-His-Pro-lle (CGP42112, 10^{-8} M (Sigma Aldrich; S-(+)-1-[(4-(dimethylamino)-3-methylphenyl)methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1*H*-imidazo [4,5-c]pyridine-6-carboxylic acid di(trifluoroacetate) salt hydrate (PD123,319, 10^{-7} M to 10^{-5} M; Sigma Aldrich); sodium nitroprusside (SNP, 10^{-7} M; Sigma Aldrich); 8-bromoguanosine 3′, 5′-cyclic monophosphate sodium salt n-hydrate (8Br-cGMP, 10^{-3} M; Wako Pure Chemical Industries, Tokyo, Japan); and 1*H*-[1,2,4]oxadiazolo [4,3-a]quinoxalin-1-one[ODQ] 1*H*- [1,2,4] oxadiazolo [4,3-a] quinoxalin-1-one (ODQ, 10^{-4} M; Sigma Aldrich). The substances were added to the cells starting on the second day for 4 days, and the medium was replaced every day. Cells were treated with the antagonist PD123,319 for 30 min before agonist treatment.

2.3. Immunocytochemical experiments

Neurite outgrowth of DRG cells was evaluated as previously reported [14]. Briefly, after treatment with drugs for 4 days, the cells were fixed with 10% formalin, and the cells were incubated overnight with rabbit anti-CGRP primary antibody (1:500, Enzo Life Sciences International, Inc., PA, USA) at 4 °C. After washing, the cells were incubated for 60 min at room temperature with HRP-labeled goat anti-rabbit IgG (Santa Cruz Biotechnology, Inc., CA, USA), diluted 1:1000 in PBS and containing 1% bovine serum albumin. Following several washes in PBS, the cells were incubated in diaminobenzidine solution.

To quantify neurite outgrowth, images from randomly chosen fields of neuronal cultures were obtained with an Olympus IX50 camera (Olympus, Tokyo, Japan). To assess neurite numbers, a 120 μm-diameter circle was pulled around the perikaryon of a single neuron, and the number of CGRP-positive neurites that crossed the drawn circle was quantified.

2.4. Western blot analysis

For western blot analysis, DRG were isolated from mice and cultured with DMEM and 10% fetal bovine serum. After 4 days in culture, the medium was replaced by serum-free DMEM. DRG cells treated under several conditions (see Section 3) and rinsed twice with PBS, followed by incubation with lysis buffer [50 mM Tris-HCl (pH = 7.6), 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% sodium lauryl sulfate and 1% Nonidet P-40]. The cell lysate samples (5 μg) were loaded onto 10% SDS-acrylamide gels. After transfer to polyvinylidene difluoride membranes (Hybond P; GE Healthcare, Japan Tokyo), the membranes were blocked in a blocking buffer: Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBS-T) and 2.5% membrane blocking agent (GE Healthcare) at room temperature for 1 h. The membranes were then probed overnight at 4 °C with rabbit anti-guanylate cyclase alpha subunit polyclonal antibody (Cayman Chemical, MA, USA, 1:3000) in blocking buffer. After the membranes were washed in TBS-T, the membranes were subsequently incubated with goat anti-rabbit antibody HRP-linked IgG (Santa Cruz Biotechnology, Santa Cruz, CA, 1:5000) in blocking buffer for 1 h at room temperature. The polyvinylidene difluoride membranes were then washed with TBS-T, and the bound antibodies were detected using a chemiluminescent substrate kit (GE Healthcare). Bands were analyzed by densitometry using FluorchemTM8800 (Alpha Innotech, San Leandro, CA, USA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression, which was detected by rabbit anti-GAPDH antibody (1:10,000; Sigma Aldrich) was used as a control to detect soluble guanylate cyclase (sGC) to ensure that the same amount of protein was loaded in each lane.

2.5. RNA extraction

Total RNA was extracted from DRG neurons, placed in RNAlater (Life Technologies, Tokyo, Japan), and stored at −30 °C. Total RNA was extracted using the RNeasy Plus Micro kit (Qiagen, Tokyo, Japan). The RNA samples were subsequently dissolved in nuclease-free water (Qiagen); the optical density values of each sample were determined using an absorption meter (Shimadzu Co., Tokyo, Japan). We performed reverse transcription using M-MLV (Wako Pure Chemical Industries, Ltd., Osaka, Japan) according to the manufacturer protocol. Specificity of amplification was verified by monophasic characteristics of the melting curve generated for each amplification product by the Eco Real-Time PCR System (Illumina Inc., Tokyo, Japan).

2.6. Quantitative analysis by real-time PCR

The reverse-transcribed mixture was used as a template for subsequent real-time PCR. Real-time PCR was performed according to the KAPA SYBR Fast qPCR kit (Nippon Genetics, Tokyo, Japan) and analyzed using an Eco Real-Time PCR System (Illumina). Primers, which were designed by the authors, were based on coding sequences of mouse genes deposited in GenBank. The data were analyzed using the mean threshold cycle equation. Primer information is shown in Table 1. GAPDH served as the internal control. The threshold cycle values for both the AT_2 receptors and internal control (GAPDH) genes were determined. The fold-change of each