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Endothelin-1 stimulates cyclin D1 expression in rat cultured astrocytes via activation of Sp1

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ABSTRACT

Endothelins (ETs), a family of vasoconstrictor peptides, are up-regulated in several pathological conditions in the brain, and induce astrocytic proliferation. We previously observed that ET-1 increased the expression of cyclin D1 protein. Thus, we confirmed the intracellular up-regulation of cyclin D1 by ET-1 in rat cultured astrocytes. Real-time PCR analysis indicated that ET-1 (100 nM) and $Ala^{1,3,11,15}$-ET-1 (100 nM), a selective agonist of the ET_B receptor, induced a time-dependent and transient increase in cyclin D1 mRNA. The effect of ET-1 was diminished by an ET_B antagonist (1 μM BQ788) or inhibitors of Sp1 (500 nM mithramycin), ERK (50 μM PD98059), p38 (20 μM SB203580) and JNK (1 μM SP600125), but not inhibitors of NF-κB (10 μM SN50 and 100 μM pyrrolidine dithiocarbamate). The binding assay for Sp1 indicated that ET-1 increased the binding activity of Sp1 to consensus sequences, and two oligonucleotides of the cyclin D1 promoter including the Sp1-binding sites diminished the effect of ET-1. Western blot analysis showed that ET-1 induced time-dependent and transient phosphorylation of Sp1 on Thr453 and Thr739 via the ET_B receptor. ET-1-induced phosphorylation of Sp1 was attenuated by PD98059 and SP600125. Additionally, ET-1 increased the incorporation of bromodeoxyuridine (BrdU) in cultured astrocytes and the number of BrdU-positive cells decreased in the presence of PD98059, SP600125 and mithramycin. These results suggest that ET-1 increases the expression of cyclin D1 via activation of Sp1 and induces astrocytic proliferation.

1. Introduction

In nerve tissues damaged by brain insults and neurodegenerative diseases, reactive astrogliosis, i.e., a phenotypic conversion to reactive astrocytes, occurs (Eddleston and Mucke, 1993; Maragakis and Rothstein, 2006; Kamphuis et al., 2012). The conversion to reactive astrocytes, which is characterized by cellular hypertrophy and increased expression of glial filaments, are known to have both beneficial and detrimental actions on damaged tissues (Karimi-Abdolrezaee and Billakanti, 2012). In damaged nerve tissues, various neurotrophic factors are produced by reactive astrocytes, which support survival and regeneration of neuronal cells (Dougherty et al., 2000; Tokumine et al., 2003). On the other hand, excess proliferation of reactive astrocytes causes glial scar formation at the site of injury and prevents axonal re-generation, thereby acting as a physical barrier (Sofroniew, 2009). Administration of cell cycle inhibitors were shown to reduce astrogliosis and neuronal degeneration in rat traumatic brain injury and ischemia models (Di Giovanni et al., 2005; Zhu et al., 2007), suggesting the detrimental action of excessive proliferation of reactive astrocytes in the damaged brain. In contrast, some studies suggested that the extent of the lesion was larger and neural damages were more severe in the absence of astrocytic proliferation (Faulkner et al., 2004; Myer et al., 2006). Thus, modulation of reactive astrogliosis is thought to be a potential therapeutic target for neurological disorders, and mechanisms underlying reactive astrogliosis have been investigated (Hamby and Sofroniew, 2010; Kang and Hébert, 2011). Cell proliferation is regulated by cell cycle-dependent expression of cyclin proteins. Expression of cyclin D, a member of the G1 cyclin family, is increased by mitogenic signals and promotes G1/S cell cycle transition. Increased expressions of cyclin D1 and D3 in astrocytes was observed in damaged nerve tissues and an excitotoxic insult (Di Giovanni et al., 2005; Gangoso et al., 2012; Wu et al., 2012). Reduction of reactive astrogliosis induced by brain ischemia was reported in cyclin D1 knockout mice (Zhu et al., 2007). These findings indicate an important role for cyclin D in the proliferation of reactive astrocytes in the damaged brain.

Abbreviations: BrdU, bromodeoxyuridine; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated kinase; ET, endothelin; FBS, fetal bovine serum; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; JNK, c-Jun N-terminal kinase; MEM, minimum essential medium; NF-κB, nuclear factor-kappaB; PDTC, pyrrolidine dithiocarbamate; PI, propidium iodide; Sp1, specificity protein 1; MAPK, mitogen-activated protein kinase.

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Endothelins (ETs), vasoconstrictor peptides, have multiple actions other than vascular constriction in nonvascular tissues including central nervous tissues (Goto et al., 1996; Dashwood and Loesch, 2010). The production of brain ETs is up-regulated in several pathological conditions and is involved in the pathological response of damaged nerve tissues (Nie and Olsson, 1996; Hama et al., 1997; Schinelli, 2011; Koyama et al., 2012). Receptors for ETs are classified as ET_A or ET_B types, and the brain ET_B type is predominantly expressed in astrocytes (Rogers et al., 2003). The brain ET_A type is predominantly expressed in endothelial cells although astrocytes also expressed (Schinelli et al., 2001). Previous studies showed that ETs are involved in the induction of astrogliosis. Administration of an ET_B agonist in the rat brain increased the number of reactive astrocytes (Ishikawa et al., 1997; Koyama et al., 2003; Koyama and Michinaga, 2012). Moreover, ET_B antagonists reduced astrocytic proliferation in animal brain injury models (Koyama et al., 1999; Gadea et al., 2008). Although several studies have investigated the signal mechanism of astrocytic proliferation by ETs (Supattapone et al., 1989; Gadea et al., 2008; Herrero-González et al., 2009), we previously focused on expression of cyclin D1 by ET-1 (Koyama et al., 2004). In cultured astrocytes, activation of ET_B receptors stimulated the proliferation accompanied with increased expression of cyclin D1 protein (Teixeira et al., 2000; Koyama et al., 2004). In the present study, intracellular signal mechanisms mediated between ET receptors and cyclin D1 expression were examined in rat cultured astrocytes. We found that ET-1 stimulated the transcription of cyclin D1 through activation of specificity protein 1 (Sp1), a transcription factor.

2. Materials and methods

2.1. Preparation of rat primary cultured astrocytes

All experimental protocols conformed to the Guiding principles for the care and use of animals by the National Institutes of Health guide (NIH Publications No. 80-23) and were approved by the Animal Experiment Committee of Osaka Ohtani University. Astrocytes were prepared from the cerebrum of 0- to 2-day-old Wistar rats as described previously (Koyama et al., 2012). The isolated cells were seeded at 1×10^4 cells/cm^2 in 75 cm^2 culture flasks and grown in Eagle's minimum essential medium (MEM) supplemented with 10% (v/v) fetal bovine serum and 200 mM L-glutamine. To remove small process-bearing cells on the protoplasmic cell layer (mainly oligodendrocytes progenitors and microglia), culture flasks were shaken at 250 rpm overnight, 10–14 day after seeding. Monolayer cells were trypsinized and placed onto 6-well culture plates or grass cover slips (15 mm diameter) that had been precoated with poly-lysine. At this stage, approximately 95% of cells expressed glial fibrillary acidic protein (GFAP).

2.2. Measurement of cyclin D1 mRNA levels by quantitative RT-PCR

Total RNA was extracted from cultured astrocytes using an acid-phenol method followed by repeated isopropanol, and first strand cDNA was synthesized from the total RNA (1 μg) as described previously (Koyama et al., 2012). The cyclin D1 mRNA levels in each sample were determined by real-time PCR using Syber Green fluorescent probes (Toyobo, Tokyo, Japan). The following primer pairs were used.

Cyclin D1 primers:

5′-TGCTTGGGAAGTTGTGTTGG-3′ and 5′-AATGCCATCACGGTCCCTAC-3′

Glyceraldehyde-3-Phosphate Dehydrogenase (G3PDH) primers:

5′-CTCATGACCACAGTCCATGC-3′ and 5′-TACATTGGGGGTAGGAACAC-3′

As a standard for the copy number of the PCR products, serial concentrations of each PCR fragment were amplified in the same manner. The amount of cyclin D1 was calculated as the copy number of each reverse-transcription product equivalent to 1 μg total RNA and normalized by the value for G3PDH.

2.3. Immunoblotting

Prior to experiments, astrocytes in 6-well plates were incubated in serum-free MEM for 48 h. These cells were then incubated with agents in serum-free MEM. After treatment, cells were lysed in 60 μl of cell lysis buffer (20 mM tris/HCl, pH 7.4, 150 mM NaCl, 1% (v/v) NP-40, 0.5% (v/v) deoxycholic acid, 0.1% (w/v) SDS, 0.5% (w/v) EDTA, 0.5 mM Na_3VO_4, 10 mM NaF, 2 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin) at 4 °C. The lysates were homogenized and centrifuged at 15,000 rpm for 10 min. The supernatant was collected and the protein contents were measured. The cell lysates were electrophoresed on an SDS–polyacrylamide 7.5% gel and electroblotted onto PVDF membranes. These membranes were probed with primary antibodies against phospho-T453-Sp1 (rabbit polyclonal, 1:1000, Abcam, Tokyo, Japan), phospho-T739-Sp1 (rabbit polyclonal, 1:1000, Assay biotech, CA, USA), Sp1 (rabbit polyclonal, 1:2000, Abcam), cyclin D1 (A-12 mouse monoclonal, 1:1000, Santa Cruz Biotechnology, CA, USA) and β-actin (C4 mouse monoclonal, 1:5000, Millipore, Temecula, CA, USA), and these membranes were incubated with peroxidase-conjugated secondary antibodies. The exposed X-ray films were scanned, and the densities of the protein bands were measured using Image J (NIH image 1.45).

2.4. Immunocytochemistry for GFAP and phospho-Sp1

After astrocytes on φ15 mm glass cover slips were incubated in serum-free MEM medium for 48 h, ET-1 (100 nM) was applied in serum-free MEM medium. ET-1-treated and non-treated cells were fixed with 3% (w/v) paraformaldehyde, and then incubated with a rabbit antibody against phospho-T453-Sp1 (1:200) or phospho-T739-Sp1 (1:200). To identify astrocytes, an anti-GFAP mouse monoclonal antibody (1:400, Sigma, St. Louis, MO, USA) was included in the incubation with primary antibodies. After the incubation with primary antibodies, cells were labeled by FITC-conjugated anti-rabbit IgG and rhodamine-conjugated anti-mouse IgG. In the incubation with secondary antibodies, 5 μg/mL 4′,6-diamidino-2-phenylindole (DAPI) was included to label nucleus. Labeled cells were observed using an epifluorescence microscope.

2.5. Nuclear extraction from cultured astrocytes

Nuclear extraction was performed using the Trans Max™ Nuclear Extraction Kit (Genlantis, San Diego, CA, USA). After cultured astrocytes were collected from 6-well plates, nuclear extraction was performed according to the manufacturers' protocols.

2.6. DNA binding assay for Sp1

The binding assay of Sp1 protein to the DNA consensus sequence was performed using an ELISA (enzyme-linked immunosorbent assay)-based kit (TransAM™ Sp1 transcription factor assay kit, Active Motif, Carlsbad, CA, USA). After nuclear extraction from astrocytes, the binding assay was performed according to the manufacturers' protocols. To confirm the binding of Sp1 to the cyclin D1 promoter, the following oligonucleotide pairs were used as a competitor in some experiments. Competitor (1) and (2) include Sp1-binding sites in the 5′-region of the rat cyclin D1 promoter (Kitazawa et al., 1999).

Competitor (1):