Biochemical and Biophysical Research Communications 383 (2009) 509–512

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Endothelin promotes neurite elongation by a mechanism dependent on c-Jun N-terminal kinase

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ARTICLE INFO

Article history:
Received 14 April 2009
Available online 23 April 2009

Keywords:
Neuron
Neurite
Outgrowth
Vascular
Regeneration
MAPK
Endothelin

ABSTRACT

Endothelin (ET), which is known as a vasoconstrictive peptide, is associated with a lot of biological functions. Although endothelin receptors are expressed in the central nervous system (CNS), little is known about the effects of endothelin on neuronal function. In this study, we reported that endothelins elongate cortical neurites via the endothelin A receptor. All the endothelin isoforms tested, endothelin-1, endothelin-2, and endothelin-3, promoted neurite elongation. ET-1-induced neurite elongation was specifically inhibited by treatment with BQ123, an antagonist for the endothelin A receptor. In addition, inhibition of ET-1-induced c-Jun N-terminal kinase (JNK) activation by treatment with SP600125, a JNK inhibitor, also prevented the ET-1-mediated promotion of neurite elongation. Thus, endothelin induces cortical neurite elongation through the endothelin A receptor by a mechanism dependent on JNK.

Introduction

Endothelin (ET) is a vasoconstrictive peptide with 3 isoforms, namely, ET-1, ET-2, and ET-3 [1,2]. ETs are produced from various types of cells, including vascular endothelial cells and smooth muscle cells. ET isoforms act through 2 G-protein-coupled receptors: the endothelin-A (ET_A) and endothelin-B (ET_B) receptors [3,4]. While the ET_A receptor stimulates the Gs and Gq proteins, the ET_B receptor stimulates the Gi and Gq proteins [4–6], thereby modulating intracellular signaling pathways. Each ET isoform can act through both of the receptors; however, the receptors have different affinities for the 3 isoforms. The ET_A receptor has greater affinity for ET-1, and the ET_B receptor can bind to all 3 peptides equally [3,4]. The distinction between ligand binding and receptor activation is considered to contribute to the diversity of physiological responses [5,7]. These responses induced by ETs include the regulation of the cardiovascular system as well as the modulation of the biological functions [8]. Multiple intracellular signals downstream of ETs and their receptors may also explain diverse functions of ETs.

ET has been shown to activate the c-Jun N-terminal kinase (JNK), one of the mitogen-activated protein kinases (MAPKs) [9–11]. JNK activation regulates several transcription factors, e.g., c-Jun and AFT, thereby modulating some aspects of cell morphology, including neurite elongation [12], and neuronal stress response and subsequent apoptosis [13]. It was reported that ET-JNK signaling regulates the neuronal migration of neural progenitor cells in the CNS [11]. ET guides peripheral sympathetic axons *in vivo* during the developmental stages [14]. Further, activation of JNK is required for regeneration of the injured axons of *Drosophila* [15]. These findings prompted us to hypothesize that ETs might regulate neurite behavior in a JNK-dependent mechanism.

Neurite elongation is an essential process in the formation of the neuronal circuitry and the subsequent development of neuronal function. Neurotrophic factors and guidance molecules regulate these processes [16,17]. Here we add a new member that promotes neurite elongation. We reported that ET promotes cortical neurite elongation via the ET_A receptor, and that ET-induced JNK activation was necessary for this effect.

Methods

Primary culture of cortical neurons. All the experiments were conducted in accordance with the guidelines laid down by the animal welfare committees of Osaka University. The primary cultures of cortical neurons were prepared from the cerebral cortexes obtained from postnatal day 0 or 1 Wistar rats (SLC, Shizuoka, Japan).

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doi:10.1016/j.bbrc.2009.04.085

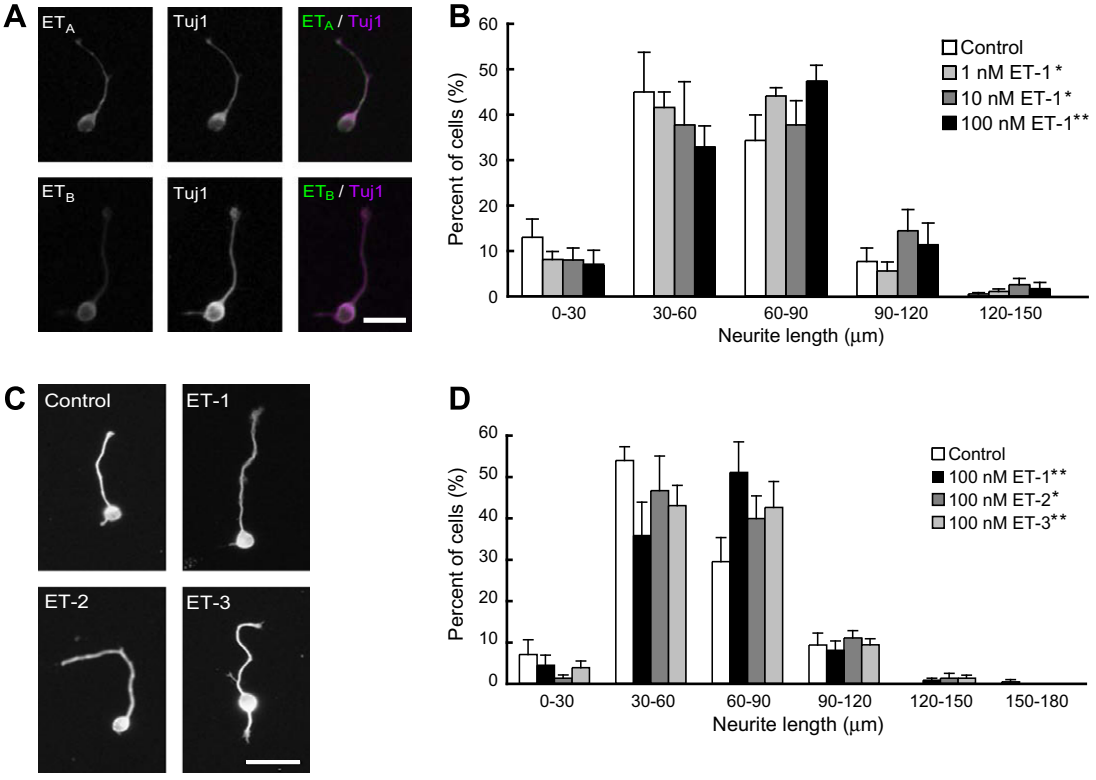

Fig. 1. The effect of endothelin-1 on neurite growth. (A) Expression of each endothelin receptor [ET_A and ET_B] in the Tuj-1-positive cultured cortical neurons was examined by immunostaining analysis. The scale bars indicate 50 μm. (B) Distribution histogram of the lengths of neurites treated with various doses of endothelin-1 (ET-1) (1, 10, and 100 nM) for 24 h. ET-1 increased the ratio of the cortical neurons with long neurites. Results are the means ± SEM of three independent experiments. (C and D) The neurite-elongation effect induced by each ET isoform was examined in cultured cortical neurons. All ET isoforms (100 nM) promoted neurite elongation of cultured cortical neurons. Results are the means ± SEM of three independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the control (n = 250–322 neurons; Kolmogorov–Smirnov test).

The cortical cells from rat pups were dissected and treated with 0.25% trypsin in phosphate-buffered-saline (PBS) for 15 min at 37 °C; then, the reaction was stopped by incubation in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS; MP Biomedical, Irvine, CA), which was followed by washing with the serum-containing medium. For the neurite-outgrowth assay, the isolated cells were plated on poly-L-lysine-coated Lab-Tek 4-well chamber slides at a density of 1–2 × 10^4 cells/cm^2 in DMEM containing 10% FBS.

Pharmacological treatment. After plating the neurons, each ET isoform (ET-1, ET-2, and ET-3; Sigma, St. Louis, MO) was added to the cultures. The MAPK activation was inhibited by treating the cells with MEK inhibitor U0126 (1 μM; Sigma, St. Louis, MO), JNK inhibitor SP600125 (1 μM; Calbiochem, La Jolla, CA), or p38 MAPK inhibitor (1 μM; Sigma). In order to determine the involvement of the ET receptors, the cultures were pretreated for 20 min with the ET_A-receptor antagonist BQ123 (1 μM; Calbiochem) or the ET_B-receptor antagonist BQ788 (1 μM; Calbiochem), which was followed by stimulation with each ET for 24 h.

Immunostaining and observation. The cultures were fixed with 4% paraformaldehyde for 20 min at 37 °C, rinsed with PBS, permeabilized using 0.1% Triton-X-PBS for 1 h at room temperature, blocked with PBS containing 5% bovine serum albumin (BSA; minimum 98% electrophoresis grade, Sigma), and incubated with the primary antibodies in PBS containing 5% BSA overnight at room temperature. The samples were subsequently probed with the secondary antibodies for 2 h and mounted onto coverslips by using a fluorescent mounting medium (Dako, Glostrup, Denmark). We used the following primary antibodies: mouse anti-β-tubulin (Tuj1) (1:1000, Covance, Richmond, CA), rat anti-endothelin A receptor (1:100, Alexis, San Diego, CA), and rat anti-endothelin B receptor (1:100, Alexis). Alexa Fluor 488 and 568 (1:1000; Invitrogen) were used as the secondary antibodies. The images were taken with an upright microscope (Olympus, Tokyo, Japan) and analyzed using a DP-controller image system (Olympus). The neurite lengths were measured using ImageJ. The Kolmogorov–Smirnov test was performed to compare the neurite-length distributions. Data were pooled from at least three independent experiments.

Results and discussion

Endothelins promote growth of cortical neurons

ET-receptor expression in the CNS has been reported in the cerebral cortex [18]. To elucidate the biological function of ETs in the CNS, we investigated whether ETs exert any effects on cultured cortical neurons; in this study, we used immunostaining to investigate the expression of ET receptors in cultured cortical neurons. We were able to detect the expression of both the ET receptors in the cultured cortical neurons (Fig. 1A). Then, we cultured cortical neurons in the presence or absence of ET-1 (1, 10, and 100 nM) in order to test the possibility that ET-1 promotes neurite elongation. We cultured the cells for 24 hours *in vitro* and measured the length of the Tuj1-positive neurites in the neurons that were immunostained using the anti-Tuj1 antibody, a neuronal marker. We prepared a distribution histogram of the neurite lengths and compared it with the distribution changes occurring after culturing with ET-1. The histogram of neurite lengths in the neurons cultured with ET-1 showed a different pattern in comparison with that observed in case of the control, indicating that the ET-1-primed cortical neurons grew longer neurites (Fig. 1B). This effect