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Full paper

Prostaglandin E2 facilitates neurite outgrowth in a motor neuron-like cell line, NSC-34

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ABSTRACT

Prostaglandin E2 (PGE2) exerts various biological effects by binding to E-prostanoid receptors (EP1-4). Although recent studies have shown that PGE2 induces cell differentiation in some neuronal cells such as mouse DRG neurons and sensory neuron-like ND7/23 cells, it is unclear whether PGE2 plays a role in differentiation of motor neurons. In the present study, we investigated the mechanism of PGE2-induced differentiation of motor neurons using NSC-34, a mouse motor neuron-like cell line. Exposure of undifferentiated NSC-34 cells to PGE2 and butaprost, an EP2-selective agonist, resulted in a reduction of MTT reduction activity without increase the number of propidium iodide-positive cells and in an increase in the number of neurite-bearing cells. Sulprostone, an EP1/3 agonist, also significantly lowered MTT reduction activity by 20%; however, no increase in the number of neurite-bearing cells was observed within the concentration range tested. PGE2-induced neurite outgrowth was attenuated significantly in the presence of PF-0441848, an EP2-selective antagonist. Treatment of these cells with dibutyryl-cAMP increased the number of neurite-bearing cells with no effect on cell proliferation. These results suggest that PGE2 promotes neurite outgrowth and suppresses cell proliferation by activating the EP2 subtype, and that the cAMP-signaling pathway is involved in PGE2-induced differentiation of NSC-34 cells.

1. Introduction

Motor neurons whose cell bodies are present in the central nervous system (CNS) control contraction of skeletal muscle.[1] Motor neurons are classified broadly into two types: upper and lower motor neurons. In particular, lower motor neurons found in the ventral horn of the spinal cord are unique cells that are directly transfer neuronal signals from the CNS to peripheral skeletal muscle. In developing motor neurons, regulation of the balance between cell proliferation and differentiation is needed for maturation of these neurons. Indeed, motor neuronal progenitor cells isolated from mice spinal cords differentiate into mature motor neurons efficiently when they exit the cell cycle.[2] Damage to lower motor neurons results in a characteristic set of diseases known as motor neuron diseases (MND), which include amyotrophic lateral sclerosis (ALS), primary lateral sclerosis and spinal muscular atrophy (SMA). Motor neuron progenitors and differentiated motor neurons from affected patients have been used to obtain human induced pluripotent stem (iPS) cells, which have been employed to clarify the pathogenesis of these diseases and to develop a form of transplantation therapy.[3,4] Therefore, highly efficient differentiation of progenitor cells and iPS cells into motor neurons is important to pave the way for the development of new medicines and regeneration therapy.

Prostaglandin E2 (PGE2) is one of the major lipid mediators produced by the arachidonic acid cascade. Arachidonic acid liberated from cellular membranes by phospholipases is converted to prostaglandin H2 (PGH2) by cyclooxygenase (COX)-1 and -2. Subsequently, and thereafter, PGH2 is converted to PGE2 by microsomal and cytosolic PGE synthase.[5] PGE2 is involved in various

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physiological effects such as inflammation,[6] pain sensitization,[7] fever[8] and wakefulness.[9] More recently, PGE2 has also been recognized to play a pivotal role in tissue maintenance and regeneration.[10] These divergent effects of PGE2 are exerted through binding to four distinct E-prostanoid receptors (EP1-4), which are coupled to G-proteins.[11] It has been reported that EP1 is involved in mobilization of intracellular Ca^{2+}, that EP2 and EP4 upregulate the intracellular levels of cyclic adenosine monophosphate (cAMP) through activation of adenyl cyclase (AC) via stimulatory G (Gs) protein, and that EP3 decreases or increases intracellular cAMP levels by activating inhibitory G (Gi) protein or Gs protein, respectively.[11] It has been shown that the COX-2 inhibitors, meloxicam and nimesulide, cause suppression of neurogenesis in the hippocampus and the subventricular zone (SVZ) of adult mice.[12] Furthermore, previous studies have found that PGE2 can enhance neuronal differentiation in a variety of neuroblastoma cell lines such as NG108-15 mouse neuroblastoma x rat glioma hybrid cells[13] and human neuroblastoma SK-N-BE(2)C cells,[14] and that neuritogenesis of cultured mouse dorsal root ganglion (DRG) neurons[15] and sensory neuron-like ND7/23 cells[16] is modulated by PGE2 signaling. In spite of growing evidence for the role of PGE2 signaling in the development of neurons in the brain, the effects of PGE2 on differentiation and neuritogenesis of motor neurons remain largely unknown.

NSC-34 is a hybrid cell line produced by fusion of motor neuron-enriched spinal cord cells with mouse neuroblastoma NG18TG2 cells.[17] These cells can be differentiated in culture medium containing low serum concentrations with extended neurites, a morphological marker of neuronal cell maturation and differentiation, and thereby these differentiated NSC-34 cells can be employed as an experimental model of motor neuron-like cells in view of their primary motor neuron-like physiological characteristics.[17,18] Moreover, we have reported previously that EP2 and EP3 are highly expressed in NSC-34 cells as well as motor neurons in the mouse spinal cord.[19] These results suggest that NSC-34 cells would be a suitable model for assessing the effects of PGE2 on cell maturation and differentiation in motor neurons.

In the present study, we sought to elucidate the mechanism of PGE2-induced differentiation of motor neurons using NSC-34, a mouse motor neuron-like cell line. We found that exogenously applied PGE2 and an EP2 agonist, butaprost, suppressed cell proliferation and promoted neurite outgrowth, and that a cAMP analog facilitated neurite outgrowth with no effect on the proliferation of these cells.

2. Materials and methods

2.1. Cell culture and reagents

The motor neuron-like cell line NSC34 (provided by Dr Neil Cashman, University of Toronto, Toronto, ON, Canada) were maintained in Dulbecco's modified Eagle Medium (DMEM; Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific Inc, Waltham, MA, USA), 100 units/mL penicillin (Thermo Fisher Scientific Inc), 100 μg/mL streptomycin (Thermo Fisher Scientific Inc) in a humidified atmosphere containing 5% CO2 at 37 °C. Cultures were used 5–15 passages. NSC-34 cells were seeded at a density of 4.0×10^3 cells/well in 96-well plate (Asahi Techno Glass, Tokyo, Japan) or 2.5×10^4 cells/well in 24-well plates (Asahi Techno Glass). PGE2 (Sigma), butaprost (Cayman Chemicals, Ann Arbor, MI, USA) and sulprostone (Cayman Chemicals) were dissolved in ethanol. PF-04418948 (Cayman Chemicals) and L-798,106 (Cayman Chemicals) were dissolved in dimethyl sulfoxide. Dibutyryl-cAMP (Daiichi Sankyo, Tokyo, Japan) was dissolved in distillated water.

2.2. MTT reduction assay

Proliferative potential of NSC-34 cells was determined using a [3-(4, 5)-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium (MTT) reduction assay as described previously.[20,21] Briefly, the NSC-34 cells were incubated with MTT (0.25 mg/mL) for 3 h at 37 °C, and the reaction was stopped by adding a solution of 50% N, N-dimethylformamide and 20% SDS, pH 4.8. The next day, the amount of MTT formazan product was determined by measuring its absorbance with a microplate reader at a test wavelength of 570 nm and reference wavelength of 655 nm.

2.3. Quantification of neurite outgrowth

Neurite outgrowth analysis was performed according to the method described by Oda et al.[22] In brief, 50 cells per condition were randomly chosen for counting neurite-bearing cells using phase-contract microscopy (IX71, Olympus, Tokyo, Japan). Neurite outgrowth was quantified as the percentage of cells bearing neurite processes >1 cell diameter in length.

2.4. Propidium iodide stain

Cell mortality was analyzed using a nuclear staining assay.[20] Before an incubation period, the propidium iodide (PI; Thermo Fisher Scientific Inc) dyes were added to culture medium (finally 25 μg/mL for 30 min). The images were collected with inversed fluorescence microscope (IX70, Olympus, Tokyo, Japan). Cell mortality was quantified by expressing the number of PI-positive cells as a percentage of the number of cells counted using phase-contract microscopy. In a blind manner, at least 50 cells per condition were counted using image-processing software (Scion imaging software, Scion, Frederick, MD, USA).

2.5. Western blotting

The western blot analysis was performed as described previously.[19] Briefly, protein extracts were subjected to SDS-polyacrylamide gel and transferred to polyvinilidene difluoride membranes (Millipore, Billerica, MA, USA). The membrane was treated with a monoclonal antibody against caspase-3 (1:1000, Cell Signaling Technology, Danvers, MA, USA) overnight at 4 °C. After washing, the membranes were incubated with a secondary antibody (Santa Cruz Biotechnology, Dallas, TX, USA) for 1 h. Immunoreactive bands were detected by Enhanced ChemiLuminescence (GE Healthcare Life Sciences, St Chalfont, Giles, USA).

2.6. LDH assay

After NSC-34 cells were incubated with PGE2 for 48 h in 96-well plates, the supernatants of NSC-34 cells were collected in new plates and then LDH assay was performed using LDH-Cytotoxic Test wako (Wako Pure Chemical Industries, Osaka, Japan).

2.7. Statistical analysis

All the data are expressed as mean ± SEM. Statistical significance was assessed by Student's *t*-test or one-way analysis of variance (ANOVA) followed by post hoc Dunnett's multiple test, where $p < 0.05$ was considered to indicate statistical significance.

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