Electrical Stimulation Control Nerve Regeneration via the p38 Mitogen-activated Protein Kinase and CREB

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K. Kawamura: None. Y. Kano: None.

LB Qualifying Statement: This result is challenging. It holds the key to further advances in the clinical management of muscle dysfunction and sarcopenia.

Introduction: Electrical stimulation as rehabilitation management of peripheral nerve injury and axonal degeneration has been in clinical use, but its effects on promoting nerve regeneration remain controversial. Recent studies have indicated that p38 mitogen-activated protein kinase (MAPK) appears to be involved in a variety of functions, including regeneration and nervous functions [1]. However, the precise function of p38 MAPK in cellular signaling is still unknown. To elucidate the functions of p38 MAPK, we investigated the role of the p38 MAPK pathway in electrical stimulation-induced neurite outgrowth of PC12 mutant cells in which nerve growth factor (NGF)-induced neurite outgrowth is impaired.

Methods: We used PC12 mutant cell line (PC12m3) that lack induction of neurite outgrowth despite showing normal sustained activation of ERK by NGF treatment[2]. PC12m3 cells treated with NGF showed enhancement of neurite outgrowth in response to various stimulants, including electrical stimulation. The cells also exhibited sustained activation of p38 MAPK induced by various stimulants. For experiments on neuritogenesis, the cells were plated in 25 cm² flasks at a density of 2 - 5x10⁵ cells per dish of serum-containing DMEM and then treated with NGF and/or exposed to electrical stimulation for 5 to 60 min using 100 mA electric current. After 7 days of incubation, the lengths and numbers of neurites were measured. Cells possessing at least one neurite with a length at least 1.5-fold greater than the diameter of the cell body were counted as previously described [2]. Each value is the mean ± S. D. for 100 - 200 cells sampled from three independent experiments. MAPK activity was determined as described previously. Briefly, PC12 and PC12 mutant cells were plated at a density of 1 x 10⁶ cells / 25 cm² in a flask of serum-containing medium and cultured for 3 days. Then the culture medium was replaced by 0.5% FBS-containing medium, and the cells were cultured for a further 48 h. PC12 parental and PC12m3 cells were then treated for 30 minutes with NGF (30 ng/ml) or exposed to electrical stimulation for 30 min. MAPK activity in cell lysates was then assayed. The cells were lysed in a lysing buffer. Aliquots of the lysates (10-15μg) from each sample were fractionated on SDS-10% polyacrylamide gel and transferred to polyvinylidene difluoride membranes. The blots were probed with antibodies specific for phospho-ERK1/2, phospho-p38 MAPK or total p38 MAPK at a dilution of 1:1000 in blocking buffer for 12 h at 4 degrees centigrade. The blots were probed with a secondary antibody, horseradish peroxidase-linked anti-rabbit IgG, at a dilution of 1:2000 in blocking buffer for 60 min at room temperature. The blots were stained for 1 min using the nucleic acid chemiluminescence reagent and exposed to x-ray film. To examine the role of activation of the MAPK signaling pathway in electrical stimulation-induced neurite outgrowth of PC12m3 mutant cells, the cells were pretreated for 30 min with various concentrations of SB203580, a specific inhibitor of p38 MAPK, and then stimulated with electrical stimulation.

Results: When cultures of the PC12 mutant (PC12m3) cells were exposed to electrical stimulation at 100 mA for 30 min, neurite outgrowth was greatly enhanced. The neurite extension was inhibited by the p38 MAPK inhibitor SB203580. Longer electrical stimulation of PC12m3 cells provoked cell death(Fig. 1, 2). When cultures of the PC12 mutant (PC12m3) cells were exposed to electrical stimulation at 100 mA for 30 min, activity of p38 MAPK and CREB increased. The activation of CREB was inhibited by the p38 MAPK inhibitor SB203580(Fig. 3, 4 ).
Fig. 1 Phase-contrast photomicrographs (x200) of PC12m3 cells were taken seven days after treatment of electrical stimulation in the presence of NGF (30 ng/ml).
A: Control, B: Electrical stimulation for 30 min, C: Electrical stimulation for 30 min in the presence of the p38 MAPK inhibitor SB203580, D: Electrical stimulation for 60 min.

Fig. 2 Electrical stimulation results in stimulation of neurite outgrowth in PC12m3 cells in the presence of NGF (30 ng/ml).
Discussion: Exposure of PC12 cells to NGF results in phosphorylation of Trk, which is a component of the NGF receptor, and subsequent activation of the GTP-binding protein Ras. Expression of the proto-oncogenic Ras activates the Raf-MEK-MAPK signaling pathway, which has been shown to play an important role in the induction of neurite outgrowth. Mammalian cells contain at least three MAPK pathways, which regulate the activities of extracellular signal-regulated kinase (ERK) MAPKs, stress-activated protein kinase (SAPK, also referred to as c-Jun N-terminal kinase (JNK)) MAPKs, and p38 MAPKs. While ERK is mainly activated by mitogenic stimuli, the JNK and p38 pathway are activated by proinflammatory or stressful stimuli. p38 MAPK is activated by a wide range of stresses, however, the precise function of p38 MAPK in cellular signaling is still unknown. In PC12m3 mutant cells treated with NGF, neurite outgrowth is stimulated by electrical stimulation and p38 MAPK is activated by electrical stimulation. The neurite extension was inhibited by the p38 MAPK inhibitor SB203580. The activation of CREB was inhibited by the p38 MAPK inhibitor SB203580. These findings suggest that electrical stimulation-induced activation of p38 MAPK and CREB pathway is responsible for the neurite outgrowth.
Significance: Electrical stimulation is of major importance for prevention or treatment of muscle dysfunction and sarcopenia. Axonal degeneration affects muscle density in older men and women. The effects of electrical stimulation on promoting nerve regeneration remain controversial. Therefore, understanding the molecular mechanisms of electrical stimulation on promoting nerve regeneration holds the key to further advances in the clinical management of muscle dysfunction and sarcopenia.

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