Induction of Neuritogenesis in PC12 Cells by a Pulsed Electromagnetic Field via MEK-ERK1/2 Signaling

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ABSTRACT. We examined the regulation of neuritogenesis by a pulsed electromagnetic field (PEMF) in rat PC12 pheochromocytoma cells, which can be induced to differentiate into neuron-like cells with elongated neurites by inducers such as nerve growth factor (NGF). Plated PC12 cells were exposed to a single PEMF (central magnetic flux density, 700 mT; frequency, 0.172 Hz) for up to 12 h per day and were then evaluated for extent of neuritogenesis or acetylcholine esterase (AChE) activity. To analyze the mechanism underlying the effect of the PEMF on the cells, its effects on intracellular signaling were examined using the ERK kinase (MEK) inhibitors PD098059 and U0126 (U0124 was used as a negative control for U0126). The number of neurite-bearing PC12 cells and AChE activity increased after PEMF exposure without the addition of other inducers of neuritogenesis. Additionally, PEMF exposure induced sustained activation of ERK1/2 in PC12 cells, but not in NR8383 rat alveolar macrophages. Furthermore, U0126 strongly inhibited PEMF-dependent ERK1/2 activation and neuritogenesis. The PEMF-dependent neuritogenesis was also suppressed by PD098059, but not U0124. These results suggest that PEMF stimulation independently induced neuritogenesis and that activation of MEK-ERK1/2 signaling was induced by a cell-type-dependent mechanism required for PEMF-dependent neuritogenesis in PC12 cells.

Key words: electromagnetic field, PEMF, ERK1/2, neuritogenesis, PC12 cells

Introduction

PC12 is a rat cell line derived from an adrenal pheochromocytoma and a useful model system for neurobiochemical and neurochemical studies (Radio and Mundy, 2008; Vaudry *et al.*, 2002). PC12 cells differentiate into neuron-like cells when exposed to various factors, including nerve growth factor (NGF) and bone morphogenetic proteins (BMPs) (Deutsch and Sun, 1992; Greene and Tischler,

1976; Koike *et al.*, 2006; Kudo *et al.*, 2011; Rydel and Greene, 1987).

The addition of NGF to PC12 cells causes sustained activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2), members of the mitogen-activated protein kinase (MAPK) family, through activation of NGF receptor tropomyosin-related kinase A (TrkA). Sustained activation of ERK1/2 leads to neurite outgrowth and the development of phenotypic characteristics in PC12 cells (Kao *et al.*, 2001; Rakhit *et al.*, 2001). However, the mechanisms that link the activation of NGF receptors to neuritogenesis are not well-defined.

BMPs are members of the transforming growth factor (TGF)- β cytokine superfamily, which mediates multiple

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biological processes, including neuronal development (Wrana *et al.*, 1994). BMPs bind to two classes of transmembrane receptors (Hogan, 1996) and activate two downstream pathways, the Smad and p38 MAPK signaling pathways (Heldin *et al.*, 1997; Kimura *et al.*, 2000). BMPs have also been shown to induce neurite outgrowth in PC12 cells and neurons (Fantetti and Fekete, 2012; Iwasaki *et al.*, 1996). In the BMP-induced neuronal differentiation of PC12 cells, neuritogenesis is dependent upon BMP-mediated p38 MAPK signaling (Iwasaki *et al.*, 1999; Yanagisawa *et al.*, 2001).

While electromagnetic fields, including pulsed electromagnetic fields (PEMFs), are considered a noninvasive way to promote and align nerve regeneration (Schimmelpfeng *et al.*, 2005), their mode of action at the cellular and molecular levels is unclear. Previous studies indicate that the biological effects of PEMFs, with or without NGF, on neuronal or neuron-like cells appear to be dependent on the usage of a PEMF (Morgado *et al.*, 1998; Schimmelpfeng *et al.*, 2005; Zhang *et al.*, 2006). Few studies have investigated the molecular mechanisms of intracellular signaling in PC12 cells subjected to PEMF stimulation alone.

In light of the possible therapeutic applications of electromagnetic fields, we examined the likelihood of controlling neuritogenesis or acetylcholine esterase (AChE) activity, characteristics of a differentiation phenotype of PC12 cells (Greene *et al.*, 1981), with a single PEMF of 700 mT, pulsed for 0.04 ms in monophasic form at 0.172 Hz alone for various time periods in PC12 cells. The PEMF used in this study induced neurite outgrowth gradually and independently in PC12 cells. Here, we report the molecular characterization of PEMF-induced intracellular signaling and neurite outgrowth in PC12 cells and discuss a possible mechanism of action of PEMF.

Materials and Methods

Cells and reagents

PC12 cells and NR8383 rat alveolar macrophages were provided by RIKEN BRC and ATCC, respectively. Recombinant human BMP4 (Peprotech) and recombinant human β -NGF (Peprotech) were dissolved in LF6 buffer solution (5 mM glutamic acid, 5 mM NaCl, 2.5% glycine, 0.5% sucrose, 0.01% Tween 80). The MEK1/2-specific inhibitors U0126 (Calbiochem) and PD098059 (Cayman Chemical Company), and a negative control for U0126, U0124 (Merck Millipore), were dissolved in dimethyl sulfoxide (DMSO).

Antibodies

Western blot analysis was performed as described previously (Kudo *et al.*, 2011). The following antibodies were used: anti-phosphorylated (activated) ERK1/2 and anti-ERK1/2 (Cell Signaling Technology).

Cell culture and the induction of differentiation

PC12 cells and NR8383 cells were maintained as described previously (Kudo *et al.*, 2011). For neurite outgrowth assays using PC12 cells, cells were seeded in growth medium at a density of 1×10^4 , 2×10^4 , or 6×10^4 cells/well in collagen type IV-coated 6- or 24-well culture plates (BD Biosciences) and allowed to grow for 24 h. Subsequently, the cells were serum-starved in DMEM supplemented with 1% horse serum and set on the coil of an electromagnetic stimulator (IMS101-50; Institute of Field Generation). Exposure to a single PEMF (magnetic flux density at the center of the coil, 700 mT; frequency, 0.172 Hz (duration of each cycle, 5.8 s); pulse duration, 0.04 ms; pulse form, monophasic) or a double PEMF (magnetic flux density at the center of the coil, 700 mT; frequency, 0.172 Hz (duration of one cycle, 5.8 s); interval between the two pulses, 0.5 s; pulse duration, 0.04 ms; pulse form, monophasic) was performed for various time periods (0–12 h/day) for a maximum of 14 days according to the manufacturer's instructions. Alternatively, cells were treated with 40 ng/ml BMP4 or 50 ng/ml NGF as a positive control for neuritogenesis. For PEMF exposure lasting longer than 3 h, a 1-h break was programmed using a PT50DG digital timer (REVEX) to avoid overheating of the coil on the electromagnetic stimulator. Neuritogenesis was quantified by examining the cells using a DP72 phase-contrast microscope (Olympus). Three images per well were captured, and cells displaying projections 1.5 times longer than the length of the cell body were considered positive. At least 300 cells were counted per well; each data point corresponds to the counts obtained from three independent wells.

AChE assay

To evaluate AChE activity in PC12 cells, the choline produced from acetylcholine by endogenous AChE in each sample was quantified using an Amplitude Fluorimetric acetylcholine esterase assay kit (AAT Bioquest) according to the manufacturer's instructions. For each sample, the signal was read using a microplate reader (GloMax-Multi Detection System; Promega) as the absorbance at 560 nm and normalized to the protein concentration.

Statistical analysis

The data are presented as the mean $\pm$ SE. Significant differences between groups were identified by Student's *t*-test or one-way analysis of variance followed by Tukey's test. *P*-values < 0.05 were considered statistically significant.

Results and Discussion

To examine the effects of a PEMF on the neuronal differentiation of PC12 cells, we first investigated the dose effect of exposure to a single PEMF (see Materials and Methods for details) on neuritogenesis. PC12 cells were either incubated for 6 days in the presence of a positive control (40 ng/ml

Fig. 1. Dose effect of the PEMF on neurite outgrowth in PC12 cells. (A–F) PC12 cells were treated with 40 ng/ml BMP4 or 50 ng/ml NGF, exposed to a single PEMF (sPEMF) for various time periods, or left untreated for 6 days. Phase-contrast images of PC12 cells on day 6 after culture without stimulation (A), with PEMF exposure for 0.75 h/day (B), with PEMF exposure for 3 h/day (C), with PEMF exposure for 12 h/day (D), with 40 ng/ml BMP4 (E), or with 50 ng/ml NGF (F). Scale bar, 100 μm. Similar results were obtained in three independent experiments. (G, H) PC12 cells were treated with 40 ng/ml BMP4 or 50 ng/ml NGF, exposed to the PEMF for the indicated time periods, or left untreated for 6 days, and the percentage of neurite-bearing cells on day 6 was determined. The data represent the mean ± SE of three replicates. ** $P < 0.01$ vs. the day-6 controls with no stimulation.

BMP4 or 50 ng/ml NGF) or exposed to the PEMF for various time periods (a total of 0.75, 3, or 12 h per day), and the extent of neurite outgrowth was evaluated. Neurite extension was less than 3% in PC12 cells incubated without stimulation for 6 days (Fig. 1A, G). Treatment with BMP4, an inducer of neuritogenesis in neurons (Fantetti and Fekete, 2012), induced neuritogenesis gradually by day 6 (Fig. 1E, G), although the effect was not as strong as that for NGF-induced neuritogenesis (Fig. 1F, H; Hayashi *et al.*, 2001). No significant enhancement of neurite outgrowth was observed on day 6 in PC12 cells exposed to the PEMF for 0.75 or 3 h (Fig. 1B, C, G). In contrast, exposure to the PEMF for 12 h significantly induced the outgrowth of neurite-like projections in PC12 cells in the absence of other neuritogenesis inducers, similar to the results for BMP4 (40 ng/ml) (Fig. 1D, G). The morphology of the neurite-like projections generated as a result of exposure to the PEMF for 12 h resembled that of the BMP4-induced neurites (Fig. 1D, E, G) and fewer neurites were observed on day 6 in PEMF-exposed or BMP-treated neurite-bearing cells than in NGF-treated cells (Fig. 1D–F).

We next evaluated the time course of PEMF-dependent neuritogenesis in PC12 cells. We first scored PC12 cells exposed to the PEMF for 12 h for neurite outgrowth on the indicated days (days 0–7). As shown in Fig. 2A and F, prior

to exposure on day 0, the cells were relatively small and round with few visible neurites. PEMF-mediated neurite outgrowth occurred gradually in a time-dependent manner (Fig. 2B–F). These results indicate that PEMF stimulation has the potential to induce neuritogenesis independently in PC12 cells via an unknown and novel mechanism. Specifically, PEMF-dependent neuritogenesis may involve the activation of an intracellular signaling pathway.

We also assessed whether PEMF exposure increases AChE activity in the cells. Cell lysates were analyzed for AChE activity after culture for 6 or 14 days with exposure to a single PEMF (12 h/day), 50 ng/ml NGF, or 40 ng/ml BMP4. As shown in Fig. 2G, a single PEMF had no significant effect on AChE activity on day 6, in contrast to NGF. However, it significantly increased AChE activity by day 14, similar to BMP4 (Fig. 2H). These results suggest that stimulation with the PEMF gradually induces not only morphological differentiation, but also functional differentiation, in PC12 cells.

Previous work has shown that activation of the ERK1/2 signaling pathway induces neuritogenesis in PC12 cells (Kao *et al.*, 2001) and that NGF-induced ERK1/2 activation and neurite outgrowth are blocked by the MEK1/2 inhibitors PD098059 and U0126 (Ahn and Tolwinski, 1999; Pang *et al.*, 1995). Thus, we next assessed whether PEMF expo-

Fig. 2. Time course of PEMF-induced neuronal differentiation in PC12 cells. (A–E) PC12 cells were exposed to a single PEMF (sPEMF) for a 12-h period each day or left untreated for 7 days. (A) Phase-contrast images of the cells on day 0 prior to exposure to the PEMF. Phase-contrast image of PC12 cells on day 3 after exposure to the PEMF (12 h/day) (B) or no stimulation (C). Phase-contrast image of PC12 cells on day 7 after exposure to the PEMF (12 h/day) (D) or no stimulation (E). Scale bar, 100 μ m. Similar results were obtained in three independent experiments. (F) PC12 cells were exposed to the PEMF for 12 h/day or left untreated for 7 days, and the percentage of neurite-bearing cells on days 0, 1, 3, and 7 was determined. The data represent the mean $\pm$ SE of three replicates. * P <0.05, ** P <0.01 vs. the day 0 control with no treatment. (G, H) Cell lysates from PC12 cells were prepared and analyzed for acetylcholinesterase (AChE) activity after stimulation with a sPEMF (12 h/day) or positive controls (50 ng/ml NGF or 40 ng/ml BMP4) for the indicated periods of time. The data represent the mean $\pm$ SE of three replicates. †† P <0.01 vs. the day-0 control with no stimulation. n.s., not significant vs. the day 0 control with no treatment. ** P <0.01 vs. the day-6 or day-14 control with no stimulation.

sure activates the ERK1/2 signaling pathway. Cell lysates were analyzed for endogenous ERK1/2 activity at 20, 60, and 180 min after exposure to a single PEMF in the presence or absence of 2.5 μ M U0126. Next, we evaluated the time course of ERK1/2 activity by Western blotting. As shown in Fig. 3A, the induction of ERK1/2 phosphorylation was detected after 60 min of PEMF stimulation and continued up to the 180-min time point. Sustained PEMF-dependent activation of ERK1/2 was completely abolished in the presence of U0126 (Fig. 3A).

We next investigated whether PEMF exposure induces sustained ERK1/2 activation in other mammalian cells. Cell lysates from either NR8383 cells or PC12 cells (as a control) were analyzed for endogenous ERK1/2 activity at 3 h

after stimulation with a single PEMF, a double PEMF (see Materials and Methods for details), or 50 ng/ml NGF (as a positive control). Next, ERK1/2 activity was evaluated by Western blotting. As shown in Fig. 3B, ERK1/2 phosphorylation was detected after 3 h of both PEMF stimulations in PC12 cells, but not NR8383 cells, although it was not as strong as that in NGF-treated cells.

The results presented in Fig. 3 prompted us to determine whether PEMF-mediated activation of the ERK1/2 signaling pathway is required for PEMF-mediated neuritogenesis in PC12 cells. We therefore pretreated cells with the MEK1/2 inhibitor U0126 at a concentration previously shown to abolish PEMF-induced ERK1/2 activation (2.5 μ M) (Fig. 3A) prior to the 6-day induction of PEMF-dependent neuro-

Fig. 3. Sustained PEMF-induced activation of endogenous ERK1/2 in PC12 cells. (A) PC12 cells were exposed to a single PEMF (sPEMF) for 20, 60, or 180 min or left untreated in the presence or absence of the MEK1/2 inhibitor U0126 (2.5 μ M). Equal amounts of the sample proteins were immunoblotted and probed with antibodies against phosphorylated (activated) ERK1/2 (p-ERK1/2) and ERK1/2. (B) PC12 or NR8383 cells were exposed to a sPEMF or a double PEMF (dPEMF), treated with 50 ng/ml NGF (as a positive control), or left untreated for 3 h. Equal amounts of the sample proteins were immunoblotted and probed with antibodies against p-ERK1/2 and ERK1/2. Representative blots are shown. Similar results were obtained in three independent experiments. IB, immunoblot.

Fig. 4. Suppression of PEMF-induced neurite outgrowth in PC12 cells by MEK1/2 inhibitors. (A-E) PC12 cells were exposed to a single PEMF (12 h/day) or left untreated for 6 days in the presence of 2.5 μ M U0126, 2.5 μ M U0124, or 10 μ M PD098059. Representative phase-contrast images of PC12 cells on day 6 after culture with no stimulation (A), the PEMF (12 h/day) (B), the PEMF (12 h/day) plus 2.5 μ M U0126 (C), the PEMF (12 h/day) plus 2.5 μ M U0124 (D), or the PEMF (12 h/day) plus 10 μ M PD098059 (E). Scale bar, 100 μ m. Similar results were obtained in three independent experiments. (F, G) PC12 cells were exposed to the PEMF (12 h/day) or left untreated for 6 days in the presence of the indicated concentrations of U0126, U0124, and PD098059. The percentage of neurite-bearing cells on day 6 was assayed. The data represent the mean $\pm$ SE of three replicates. ^{††} P < 0.01 vs. the day 0 control with no stimulation. ^{**} P < 0.01 vs. PEMF stimulation (12 h/day) alone on day 6. n.s., not significant vs. PEMF stimulation (12 h/day) alone on day 6. PD, PD098059.

togenesis. The results presented in Fig. 4A–C show that 2.5 μ M U0126 almost completely suppressed PEMF-induced neuritogenesis in PC12 cells. We also scored PC12 cells incubated in the presence of U0126 at various concentra-

tions (0–2.5 μ M), the other MEK inhibitor PD098059 at a concentration high enough to suppress ERK1/2 activation (10 μ M) (Pang *et al.*, 1995), or a negative control for U0126, U0124 (2.5 μ M), to assess the inhibition of neurite

outgrowth. Our results indicate that U0126 reduced PEMF-mediated neuritogenesis in PC12 cells in a dose-dependent manner (Fig. 4F). In addition, PD098059, but not U0124, also inhibited PEMF-induced neuritogenesis in the cells (Fig. 4D, E, G). These results suggest that PEMF-induced neuritogenesis in PC12 cells requires sustained activation of MEK-ERK1/2 signaling.

Taken together, the results of the present study clearly indicate the essential role of MEK-ERK1/2 signaling in the PEMF-dependent neuronal induction of PC12 cells, and suggest the existence of a cell type- or cell content-dependent mechanism that mediates PEMF-induced MEK-ERK1/2 signaling. However, how a PEMF induces MEK-ERK1/2 activation and subsequent neuritogenesis in PC12 cells is unclear. In this context, our preliminary data show the TrkA tyrosine kinase inhibitor GW441756 had no significant effect on PEMF-induced neuritogenesis in PC12 cells (data not shown), implying that activation of MEK-ERK1/2 signaling may be independent of TrkA-mediated signaling.

In conclusion, we characterized a novel effect of a PEMF on the differentiation of PC12 cells. Specifically, PEMF exposure can independently induce neuritogenesis through MEK-ERK1/2 activation. Further research into the mechanisms underlying the action of the PEMF and the identification of its critical target signaling molecule(s) leading to neuritogenesis in PC12 cells will promote future application of electromagnetic fields to the regulation of neuronal differentiation.

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