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Activation of the JNK pathway by nanosecond pulsed electric fields

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ABSTRACT

Nanosecond pulsed electric fields (nsPEFs) are increasingly recognized as a novel and unique tool in various life science fields, including electroporation and cancer therapy, although their mode of action in cells remains largely unclear. Here, we show that nsPEFs induce strong and transient activation of a signaling pathway involving c-Jun N-terminal kinase (JNK). Application of nsPEFs to HeLa S3 cells rapidly induced phosphorylation of JNK1 and MKK4, which is located immediately upstream of JNK in this signaling pathway. nsPEF application also elicited increased phosphorylation of c-Jun protein and dramatically elevated *c-jun* and *c-fos* mRNA levels. nsPEF-inducible events downstream of JNK were markedly suppressed by the JNK inhibitor SP600125, which confirmed JNK-dependency of these events in this pathway. Our results provide novel mechanistic insights into the mode of nsPEF action in human cells.

1. Introduction

Application of electric fields in a very short duration is widely used in various fields of the life sciences, especially for the introduction of macromolecules into living cells by electroporation [1–4]. Recent advances in electrical sciences have enabled the production of high voltage electric pulses for extremely short periods in the range of nanoseconds, *i.e.*, nanosecond pulsed electric fields (nsPEFs) [5,6]. The discovery of the efficient induction of apoptosis using intense nsPEFs [7,8] led to the application of nsPEFs in the development of a rapidly expanding research field, which includes cancer therapy [9–11]. nsPEFs are increasingly recognized as a unique tool with potential, but the molecular mechanisms underlying nsPEF-induced biological responses are largely unknown.

Cells can respond to various extracellular stimuli by activating intracellular signals that are mediated by cascades of protein phosphorylation and controlled by feedback loops involving multiple regulatory factors. Mitogen-activating protein kinases (MAPKs) are serine/threonine kinases and play critical roles in many cellular functions. Mammalian cells possess three MAPK pathways, which engage in complicated crosstalk with one another to form a cellular regulatory network [12,13]. The c-Jun N-terminal kinases (JNKs) belong to the MAPK family, and mammalian cells possess three JNK members, which play crucial roles in the regulation of proliferation, differentiation, and metabolism in a cell type- and context-dependent manner [14–16]. Of the three mammalian JNK members, JNK1 is constitutively and ubiquitously expressed in human tissues [14].

Upon receiving external stimuli, JNK is phosphorylated by the upstream MAPK kinases, MKK4 and MKK7, to yield the enzymatically active form [14,17]. The activated JNK subsequently phosphorylates the N-terminal region of c-Jun [18], which is a proto-oncogene product. c-Jun exists as a heterodimer with other proteins, and one of the major interacting partners for c-Jun is c-Fos, which is also known to be a proto-oncogene product [19]. Upon phosphorylation of c-Jun by JNK, the c-Jun/c-Fos complex activates temporal expression of a distinct set of genes, including the *c-jun* and *c-fos* genes, which are collectively referred to as immediate early genes [20]. Thus, activation of JNK leads to the induction of the phosphorylation of c-Jun protein and an increase in *c-jun* and *c-fos* mRNAs. Activation of the JNK pathway also induces MAPK phosphatases, which can dephosphorylate the activated JNK and thereby attenuate the induction of the JNK pathway to serve as a negative feedback control [21].

In this study, we analyzed the activation of the JNK pathway in human HeLa S3 cells as a model for studying nsPEF action on human cells. Application of nsPEFs elicited the rapid induction of JNK1 phosphorylation, which was accompanied by activation of upstream and downstream events in the signaling pathway involving JNK. Treatment with SP600125, a potent inhibitor of JNK kinase activity, significantly suppressed nsPEF-inducible downstream events in the JNK pathway. Our results provide novel insights into the molecular mechanisms underlying nsPEF-induced biological responses.

Abbreviations: nsPEF, nanosecond pulsed electric field; JNK, c-Jun N-terminal kinase; MAP kinase, mitogen-activating protein kinase; MKK, mitogen-activating protein kinase kinase.

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2. Materials and methods

2.1. Cell culture and the application of nsPEFs to cells

HeLa S3 cells were grown in α-minimum essential medium (αMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin at 37 °C in a humid atmosphere of 5% CO_2. Prior to the application of nsPEFs, cells were gently detached from culture dishes by treatment with 2.5 mM EDTA that was diluted in phosphate-buffered saline. Trypsin was not used for cell detachment to rule out any possible effects of remaining trypsin in subsequent steps. The detached cells were washed with αMEM/10% FBS without antibiotics and suspended in αMEM/10% FBS without antibiotics at 2×10^6 cells/ml. The cell suspension (400 μl) was placed in an electroporation cuvette, which contained a pair of aluminum electrodes with a 4-mm gap (#5540, Molecular BioProducts). nsPEFs with a frequency of 1 Hz were generated using a pulsed power modulator (MPC3000S, Suematsu Electronics) [22] and applied to the cell suspension placed in the cuvette. Voltage and current waveforms of the electric pulse were monitored with a P6015A high voltage probe and a TDS2012S digital oscilloscope (Tektronix). In our standard experimental conditions, electric pulses with approximately 80 ns pulse width at half maximum were applied to the cell suspension (Fig. 1A). When 20 shots of nsPEFs at 1 Hz were applied to cells, the total exposure time to nsPEFs is approximately 19 s. After the nsPEF application, the cell suspension was diluted fivefold with αMEM/10% FBS and incubated at 37 °C for the required time. The treated cells were subsequently collected and snap-frozen in liquid nitrogen. As a positive control for the activation of the JNK pathway, cells were treated with 50 ng/ml anisomycin (Wako), or 312 nm ultraviolet (UV) irradiation at 100 mJ/cm^2 (the total exposure time to UV was 25 s). To inhibit JNK enzymatic activity, cells were pre-treated with 20 μM SP600125 for 30 min at 37 °C, then suspended in αMEM/10% FBS containing 20 μM SP600125 and treated with the previously described nsPEF application.

2.2. Cell viability measurement

Cell viability was measured by the MTT method using a Cell Proliferation Kit I (Roche) according to the manufacturer's instructions. Briefly, cells were plated at 1000 cells/well in a 96-well plate and incubated at 37 °C for the periods indicated in the figure. A tetrazolium solution was added to each well and the cells were further incubated for 4 h at 37 °C to allow formazan formation. Following solubilization of the cells in an acidic solution containing SDS, the conversion of tetrazolium into formazan was analyzed using a Multiskan FC microplate reader (Thermo Scientific).

2.3. Western blot analysis

Frozen cells were lysed in a buffer containing 20 mM Tris–Cl (pH 7.5), 150 mM NaCl, 1% Igepal, protease inhibitors (Complete EDTA-free, Roche), and a phosphatase inhibitor cocktail (PhosStop, Roche) on ice for 20 min. The cell lysates were cleared by centrifugation at 20,000 *g* for 10 min, and the protein concentration of each lysate was determined using a Protein Assay reagent (BioRad). SDS polyacrylamide gel electrophoresis was followed with western blotting, according to standard procedures. Antibodies against JNK and phosphorylated JNK (pT183/pT185) were obtained from BD Biosciences. Antibodies for c-Jun, phosphorylated c-Jun, and phosphorylated MKK4 were purchased from Cell Signaling Technology. An antibody for β-actin was obtained from Sigma–Aldrich. Antigen–antibody complexes were visualized by a chemiluminescence method using secondary antibodies conjugated to horse radish peroxidase (Santa Cruz Biotechnologies) and a Super Signal West Pico reagent (Thermo Scientific).

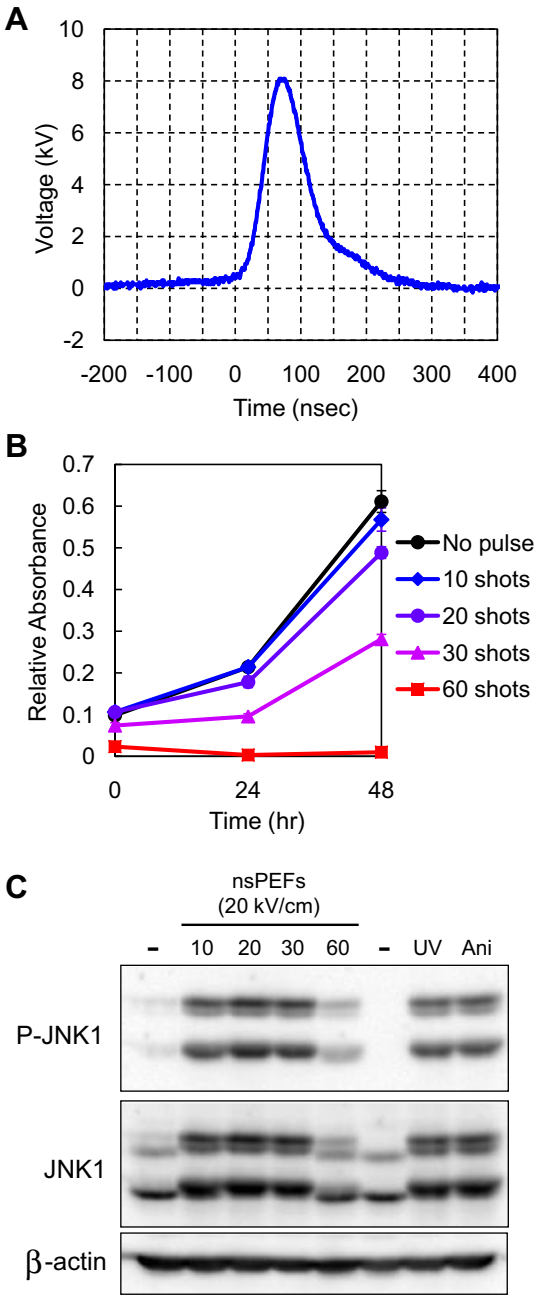

Fig. 1. Induction of JNK1 phosphorylation by nsPEFs that did not cause significant cytotoxicity. (A) Typical example of the waveform of nsPEFs. Maximum voltage of this electric pulse was 8 kV, which means 20 kV/cm electric fields in a 4 mm-gapped electroporation cuvette. The pulse width at half maximum was estimated as 80 ns. (B) Cell growth after the application of nsPEFs. The indicated shot numbers of nsPEFs at 20 kV/cm were applied to HeLa S3 cells. Cell viability at 0, 24, and 48 h after nsPEF application was analyzed by MTT assay. Average values for five independent experiments and standard deviations are shown. (C) Induction of JNK1 phosphorylation after the application of nsPEFs, ultraviolet radiation, and anisomycin. HeLa S3 cells were treated with 10, 20, 30, or 60 shots of 20 kV/cm nsPEFs and subsequently incubated at 37 °C for 15 min. Another preparation of HeLa S3 cells was treated with 312 nm ultraviolet radiation at 100 mJ/cm^2 (UV) or 50 ng/ml anisomycin (Ani). Untreated samples (–) were included as a negative control. Total cell lysates were prepared and subjected to western blot analysis using an antibody specific to the phosphorylated JNK1 (P-JNK1, *top panel*) and an antibody that reacts with JNK1 irrespective of phosphorylation (JNK1, *middle panel*). Constant amounts of proteins (30 μg) were loaded in each lane, and β-actin was detected as a loading control (*bottom panel*).