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Post-pulse addition of trans-cyclohexane-1,2-diol improves electrotransfer mediated gene expression in mammalian cells

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ABSTRACT

Electric field mediated gene transfer is facing a problem in expression yield due to the poor transfer across the nuclear envelope. Trans-cyclohexane-1,2-diol (TCHD) was shown to significantly increase chemically mediated transfection by collapsing the permeability barrier of the nuclear pore complex. We indeed observed a significant increase in expression by electrotransfer when cells are treated post pulse by a low non toxic concentration of TCHD. This was obtained for different pulsing conditions, cell strains and plasmid constructs. An interesting improvement in cell viability can be obtained. This can significantly enhance the non-viral gene electrical delivery.

1. Introduction

The plasma membrane is a barrier that hinders the transfer of molecules such as nucleic acids into cells. One physical method known to safely improve plasmid DNA delivery into cells is electropermeabilization (or electroporation) [1,2]. The application of controlled electric pulses causes a transient permeabilization of the plasma membrane and allows non permeant molecules (such as polar macromolecules) to enter the cells [1,3–5]. When the electric pulses are delivered on a cell in presence of plasmid (pDNA), gene expression can be detected [1]. This is indeed a multistep process. With the electric field pulse, pDNA are pushed against the cell membrane and pDNA/membrane aggregates are created [6,7] and remain trapped at the membrane for several minutes [6,8]. The challenge with optimizing gene electrotransfer is to get a balance between the positive contribution of creating defects supporting the cytoplasmic transfer of plasmid DNA and the need to preserve cell viability. Once it crosses the plasma membrane, pDNA is actively transported along tubulin filaments by motor proteins through the cytoplasm to the nuclear envelope [9,10] or trapped within actin coated vesicles [11,12].

The Nuclear envelope represents the last barrier that pDNA needs to cross to gain access to the transcriptional machinery. Only a very small fraction of pDNAs introduced in the cytoplasm, whatever the method, microinjection [13] as well as electrotransfection [6], can indeed reach the nuclear compartment. Furthermore pDNA present in the cytoplasm is sensitive to degradation by nucleases during the cell cycle [14]. Therefore, there is a need for a fast transfer of pDNA to the nucleus. It has been described that DNA transfection is more efficient in dividing cells compare to non-dividing cells due to the loss of nuclear envelope integrity during cell division [15–18] suggesting that the direct entry of pDNA into the nucleus requires modifications of the nuclear envelope components. One putative target is the nuclear pore complex (NPC), a multiprotein complex known to control transport through the nuclear envelope [19–21].

Different approaches have been reported to obtain such a molecular modification. One physical approach in order to destabilized NPC is to expose nuclear envelope to controlled electric pulses. Nanosecond electric pulses (nsEPs 4–600 ns) were described as being able to permeabilize internal organelles as intracellular granules, endocytic vesicles, nuclear envelope and to induce calcium release from endoplasmic reticulum (reviewed in [22]). The use of a two sequential pulses combination was proposed to improve gene transfer. Long (ms) electrical pulses were applied first as described above to allow plasmid access to cytoplasm and were followed by nanosecond electric pulses, in order to destabilize the nuclear envelope [23]. This combination was supposed to increase the number of plasmids entering the nucleus and thus, enhance gene expression. This was described as inducing a 3.6 fold increase in GFP gene expression in cells exposed to one

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nsEP 30 min after classical electrotransfection [24]. However, such a combination can be ineffective since more recently, nsEPs were shown to have no major contribution to gene electrotransfer [25]. No effect on constitutive protein expression was detected. In a similar approach, it was first shown that the application of one 5μs pulse of several kV/cm alone or after an electrotransfection protocol affects the morphology of the nucleus [26] while it was observed that such a combination of classical electrotransfection followed by one 5 μs 5 kV/cm pulse did not increase plasmid DNA expression as compared to the classical electrotransfection protocol [27]. Electrically mediated alterations of the nuclear envelope were not enhancing gene nuclear uptake.

The Nuclear Pore Complex (NPC) is composed of 30–50 different proteins (Nucleoporins), and can be, in a simplified form, divided in three basic elements: the nuclear basket, the central core and the cytoplasmic fibrils [28]. The central channel of the NPC form an aqueous channel that allow the passive diffusion of molecules smaller than 25–40 kDa across the NPC [29]. However, the wall of the NPC is rich in phenylalanine-glycine repeats (FG-nucleoporins) that act as hydrophobic barrier blocking the transfer of macromolecules such as DNA that therefore, requires active and facilitated transport [20,21]. This active transport is mediated by specific interactions between the translocating element and transport receptor with cargo via adaptor molecules. This complex association allows a highly orchestrated, rapid and efficient nuclear transport. *In vitro* studies evaluate the transport rate to 1000 cargo molecules per seconds per NPC meaning that at least 10 transport molecules cross a given NPC simultaneously [20]. Nevertheless, this process is highly selective meaning that it is dependent on the presence of Nuclear Localization Sequences (NLS) on the translocating element. These NLS allow the association of the translocating element to importin α and β promoting its transfer into the nucleus. Thus, in order to increase nuclear import of DNA, NLS-peptides and NLS-proteins have been attached to pDNA [30–32] but with limited success. This phenomenon does not seem due to size of the DNA but more to its hydrophilic properties that tend to exclude the DNA from the nuclear pore even if associated with importin.

The amphipathic alcohol *trans*-cyclohexane-1,2-diol (TCHD) has the ability to disrupt bonds between FG-nucleoporins, that makes up the NPC barrier without affecting the integrity of the nuclear envelope. Moreover, it has been described that TCHD can transiently increase the size of NPC [33] thus modifying the permeability [21]. It was shown that gene expression mediated by a chemical method (poly- and lipoplexes) was increased when the cells were treated by a low concentration of TCHD [34]. We hypothesized that a process similar would occur along electrotransfection. After the transfer of free plasmid DNA into the cell cytoplasm by electropermeabilization, treatment with TCHD could facilitate plasmid DNA access into the nucleus and thus increase gene expression. In this study we compared transfection rate between EP alone and EP combined with TCHD treatment. We also analyzed the impact of the delay between EP and TCHD treatment as well as the TCHD incubation time on gene expression and cell viability.

2. Material and methods

2.1. Cell culture

B16F10 cells were grown as a monolayer culture on T75 flasks (Nunc, Denmark) in Dulbecco's Modified Eagle Medium with 4.5 g/l D-Glucose and L-Glutamine (DMEM; Gibco/ Life technology) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St Louis, MO) and the antibiotics penicillin (100 U/ml) and streptomycin (100 U/ml) (Gibco/ Life technology) at 37 °C, 5% CO_2 atmosphere in a humidified chamber until they reached 70% confluence.

Chinese Hamster Ovarian (CHO) cells (Wild-Type Toronto) were grown as a monolayer culture in Minimum Essential Eagle Medium with Earle's salts and nonessential amino acids (EMEM; Eurobio, Les Ulis, France), supplemented with 10% fetal bovine serum (GIBCO/Life Technologies, Grand Island, NY), L-glutamine (0.58 g/l, GIBCO/Life Technologies), 2.95 g/l tryptose-phosphate (Sigma-Aldrich, St. Louis, MO), BME vitamins (Sigma-Aldrich), 3.5 g/l glucose (Sigma-Aldrich) and the *anti*biotics penicillin (100 U/ml) and streptomycin (100 μg/ml, GIBCO/Life Technologies) at 37 °C, 5% CO_2 atmosphere in a humidified chamber until they reached 70% confluence. CHO cells could grow in suspension. Plated cells were trypsinized and cultured in suspension in spinner (Corning Inc, Corning, NY, USA). 0.5×10^6/ml cells were cultured in the same culture medium in hermetic closed spinner at 37 °C with a soft stirring. Every day, the cell culture was diluted 2-fold. Growing cells in suspension avoided a trypsinization step before the delivery of electric pulses. The extracellular matrix was preserved.

2.2. Plasmid DNA

pCMV-eGFP-C1 (Clontech, Mountain View, CA) a 4.7-Kb plasmid encoding GFP, was used in experiments with B16F10 cells (map in Suppl Fig. 1). This plasmid was amplified in Escherichia coli DH5α and purified with the Maxiprep DNA Purification System (Qiagen, Germany) according to the manufacturer's protocol. The purification was verified by agarose electrophoresis.

pCMV-CpGfree-tdTomato (Invivogen, Toulouse, France) a 4.4-Kb plasmid encoding Tomato, was used in experiments with CHO cells (map in Suppl Fig. 1) and directly purchased.

2.3. Gene electrotransfection protocols and TCHD treatment

B16F10 cells were trypsinized and washed twice in medium. CHO cells were collected from spinner. Cells were suspended in pulsation buffer (PB; 10 mM K_2HPO_4/KH_2PO_4, 1 mM $MgCl_2$, 250 mM sucrose [pH 7.4]) at a concentration of 5×10^6 cells/ml, and 20 μg/ml pCMV-EGFP-C1 plasmid or 20 μg/ml pCMV-CpGfree-tdTomato plasmid was added. 100 μl of cell solution was dropped between stainless steel, flat, parallel electrodes (0.4 cm gap) in contact with the bottom of a culture dish (Nunc, Denmark). Two different electrical parameters were used: (i) Low field long pulse (LF-LP) parameters that consist in 6 square-wave pulses of 600 V/cm, duration 5 ms, (ii) High field short pulse (HF-SP) parameters consist in 4 square-wave electric pulses of 1200 V/cm, duration 100 μs (Fig. 1). All pulses were applied at 1 Hz frequency, at room temperature using a pulse generator (electrocell S20; Betatech, St Orens, France). Pulse delivery was monitored on line on the touch screen. In pulses conditions, 20 μl FBS (Sigma Aldrich, St Louis, USA) were added just after pulsing in the drop, which was hanging on the dish. Cells were kept at room temperature.

In the indicated conditions, 20 μl of TCHD (Sigma Aldrich, St Louis, USA) diluted in pulsing buffer (to get a final concentration 1% w/v), or 20 μl of pulsing buffer alone were added to the cells 0 or 10 min after pulse delivery and incubated 10, 20 or 30 min at 37 °C. Cells were then transferred to 2 ml of their respective culture medium in 12 well plates (Nunc, Denmark) and incubated 24 h at 37 °C with 5% CO_2. Cells untreated with TCHD were transferred in 2 ml culture medium 5 min after electropermeabilization (Fig. 1).