Journal of Controlled Release 228 (2016) 20–25

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Faint electric treatment-induced rapid and efficient delivery of extraneous hydrophilic molecules into the cytoplasm

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ARTICLE INFO

Article history:
Received 26 October 2015
Received in revised form 8 February 2016
Accepted 29 February 2016
Available online 2 March 2016

Keywords:
Faint electric treatment
Cellular uptake
Cytoplasmic delivery

ABSTRACT

Effective delivery of extraneous molecules into the cytoplasm of the target cells is important for several drug therapies. Previously, we showed effective in vivo transdermal delivery of naked siRNA into skin cells induced by faint electric treatment (ET) iontophoresis, and significant suppression of target mRNA levels (Kigasawa et al., Int. J. Pharm., 2010). This result indicates that electricity promoted the delivery of siRNA into cytoplasm. In the present study, we analyzed the intracellular delivery of naked anti-luciferase siRNA by faint ET, and found that the luciferase activity of cells expressing luciferase was reduced by in vitro ET like in vivo iontophoresis. Cellular uptake of fluorescent-label siRNA was increased by ET, while low temperature exposure, macropinocytosis inhibitor amiloride and caveolae-mediated endocytosis inhibitor filipin significantly prevented siRNA uptake. These results indicate that the cellular uptake mechanism involved endocytosis. In addition, voltage sensitive fluorescent dye DiBAC4 (3) penetration was increased by ET, and the transient receptor potential channel inhibitor SKF96365 reduced siRNA uptake, suggesting that faint ET reduced membrane potentials by changing intracellular ion levels. Moreover, to analyze cytoplasmic delivery, we used in-stem molecular beacon (ISMB), which fluoresces upon binding to target mRNA in the cytoplasm. Surprisingly, cytoplasmic ISMB fluorescence appeared rapidly and homogeneously after ET, indicating that cytoplasmic delivery is markedly enhanced by ET. In conclusion, we demonstrated for the first time that faint ET can enhance cellular uptake and cytoplasmic delivery of extraneous molecules.

1. Introduction

Over the last decade, the delivery of extraneous hydrophilic molecules across the plasma membrane has been a subject of intense study. For effective therapies, small chemical molecules or large biological medicines, such as functional nucleic acids, must be delivered into the cytoplasm or nucleus of target cells. The lipophilic plasma membrane presents a dynamic barrier that restricts entry of extraneous hydrophilic or charged molecules into cells, while inefficient endosomal escape is another obstacle to effective delivery of macromolecular medicines [1,2]. To overcome these cellular barriers, various strategies, including development of nanocarriers, cell penetrating peptides, and physical methods such as electroporation, have been studied [3,4]. However, given the fundamental challenges of intracellular delivery, the versatile application of these systems remains unsatisfactory due to issues with delivery efficiency and toxicity of the carrier [5,6]. Therefore, safer and more effective methods to deliver extraneous hydrophilic molecules into cells are needed.

Iontophoresis has recently attracted the attention of researchers studying drug delivery systems. Iontophoresis is the promising non-invasive transdermal drug delivery technology [7–9]. Iontophoresis facilitates transdermal delivery of water soluble and ionized molecules by application of small electrical current (0.5 mA/cm^2 or less) with electrodes on the skin surface. Thus, electric treatment of the skin provides the driving force for transdermal delivery of drug molecules. Previously, we demonstrated non-invasive in vivo transdermal delivery of naked siRNA induced by iontophoresis that significantly suppressed levels of target mRNA [10]. This result indicates that hydrophilic siRNA molecules could be delivered non-invasively into the cytoplasm of skin cells by faint electric treatment (ET) without the need for any modification of the siRNA to promote effective cellular association and endosomal escape. A mechanistic analysis of the transdermal penetration of this macromolecule revealed that ET induced the dissociation of intracellular junctions via activation of cell signaling pathways mediated by protein kinase C (PKC) [11]. However, the mechanism of cellular uptake and

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http://dx.doi.org/10.1016/j.jconrel.2016.02.048
0168-3659/

cytoplasmic delivery of siRNA and other hydrophilic molecules are still unclear. Here we explored whether faint ET could enhance cellular uptake and cytoplasmic delivery of extraneous hydrophilic molecules by affecting cellular physiology.

We examined the effect of faint ET in the presence of siRNA targeting luciferase in cells stably expressing luciferase. Uptake mechanisms were analyzed by assessing the effect of low temperature and endocytosis inhibitors such as the clathrin-mediated endocytosis inhibitor hypertonic sucrose [12], the caveolae-mediated endocytosis inhibitor filipin [13], and the macropinocytosis inhibitor amiloride [14], on the cellular uptake of fluorescent labeled-siRNA. In addition, the effect of ET on membrane potential and the effect of a transient receptor potential (TRP) channel inhibitor on fluorescent labeled-siRNA uptake were also examined. We also analyzed the cytoplasmic delivery using a functional oligonucleotide in-stem molecular beacon (ISMB), which fluoresces upon specific binding with target mRNA in the cytoplasm [15]. The present study offers ET as an effective and safe technology for cytoplasmic delivery of functional hydrophilic molecules and describes its mechanism.

2. Materials and methods

2.1. Materials

In-stem molecular beacon (ISMB) against luciferase (5′-C(Cy3:Y_D)TGG(Y_D)GTTGGCACCAGCAGCGCAC(Nitromethylred:N_m)(N_m)CCA(N_m)(N_m)G -3′: ISMB-Luc) and ISMB against GFP (5′-G(Y_D)GTT(Y_D)GAAGAAGATGGTGCGCTCTC(N_m)(N_m)AAC(N_m)(N_m)C-3′: ISMB-GFP) were synthesized by Tsukuba Oligo Service, Inc. (Tsukuba, Japan). Calcein, amiloride hydrochloride hydrate and filipin were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). SKF 96356 and sucrose were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). $DiBAC_4(3)$ was obtained from Dojindo Molecular Technologies, Inc. (Rockville, MD, USA). Lipofectamine 2000, anti-luciferase siRNA (21-mer, 5′-GCGCUGCUGGUGCCAACCCTT-3′, 5′-GGGUUGGCACCAGCAGCGCTT-3′: anti-Luc) and anti-GFP siRNA (21-mer, 5′-GCUGACCCUGAAGUUCAUCTT-3′, 5′-GAUGAACUUCAGGGUCAGCTT-3′: anti-GFP) were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). Cell lysis buffer was purchased from Promega Corporation (Madison WI, USA). The mouse melanoma cell line B16-F1 was obtained from Dainippon Sumitomo Pharma Biomedical Co, Ltd. (Osaka, Japan), and stable transformants of B16-F1 cells expressing luciferase (B16-F1-Luc) were established in our laboratory [16]. These cells were cultivated in DMEM supplemented with 10% FBS at 37 °C in 5% CO_2.

2.2. Electric treatment of the cells

For in vitro ET, cells were cultivated in 35 mm dishes. The number of cells used is mentioned in each section below. After 18 h of cultivation, cells were washed with PBS, and then 1 ml serum free DMEM containing 0.5 μg ISMB-Luc, 25 μM calcein, 100 pmol anti-Luc siRNA or anti-GFP siRNA was added to the cells. Ag–AgCl electrodes with 2.5 cm^2 surface area (3 M Health Care, Minneapolis, MN, USA) were placed into the dish, and cells were treated with a constant current of 0.34 mA/cm^2 for 15 min.

2.3. Transfection and measurement of luciferase activity

B16-F1-Luc cells were cultivated at a density of 1×10^4 cells in 35 mm culture dishes. After 18 h of incubation, cells were washed with PBS, and 1 ml serum free DMEM containing 100 pmol anti-luc siRNA was added before the cells were treated with electricity as described above. After 3 h of ET, 1 ml DMEM containing 10% FBS was added, and the cells were further incubated for 45 h. After the incubation, the cells were lysed with Reporter Lysis Buffer (Promega) according to the manufacturer's protocols. The luciferase assay substrate (Promega) was added to cell lysates, and chemiluminescence intensity was measured by a luminometer (Luminescensor-PSN, ATTO). The total protein concentration was measured with BCA protein assay kit (Thermo Scientific).

2.4. Cytotoxicity assay of ET treated cells

For determination of cytotoxicity, 4×10^5 B16-F1-Luc cells were cultivated in 35 mm culture dishes. After 18 h of cultivation, cells were washed by PBS and added 1 ml of serum free DMEM medium followed by ET for 15 min. Since the cytotoxicity by electricity-based delivery system, such as electroporation, had been observed at immediately after the treatment [17], we evaluated the cell viability at immediately after ET in this study. Immediate after ET, cells were collected from the dish by trypsin treatment, and taken 10 μl of cell suspension into a micro tube. An equal volume (10 μl) of 0.4% trypan blue solution (Wako Pure Chemical Industries, Ltd. Osaka, Japan) was added to the cell suspension, and the mixture was incubated for 2 min. Then, the numbers of stained and non-stained cells were counted. The percentage of viability was calculated by the formula (100 × number of non-stained cells / total number of cells).

2.5. Treatment with low temperature or pharmacological inhibitors and measurement of fluorescence intensity

For mechanistic studies, B16-F1 cells were seeded at a density of 1×10^5 cells in 35 mm culture dishes. After 18 h of cultivation, cells were washed with PBS. For low temperature experiments, 1 ml serum free DMEM containing 100 pmol rhodamine-labeled siRNA was added into the dish, and ET was then performed on ice. For pharmacological inhibitor experiments, 1 ml serum free DMEM containing either 0.45 M sucrose, 1 mM amiloride, 0.5 μg filipin, or 25 μM SKF96365 was added into the dish, and the dish was incubated for 15 min. After the incubation, rhodamine labeled siRNA (100 pmol) was added to the dish, and the cells were treated with electricity as described above. After the ET, the cells were incubated for 1 h at 37 °C. The cells were then lysed with reporter lysis buffer (Promega) according to the manufacturer's protocols. Fluorescence intensity of the cell lysates was measured with a microplate reader Infinite 200 (Tecan Group Ltd., Männedorf, Switzerland) at excitation and emission wavelengths of 546 nm and 576 nm, respectively.

2.6. Membrane potential measurement

To measure membrane potential, 5×10^4 cells were seeded in 35 mm culture dishes. After 18 h, cells were washed with PBS, and 2 ml DMEM containing 5 μM $DiBAC_4(3)$ dye was added before the cells were incubated for 30 min at room temperature. After incubation the cells were treated with electricity for 15 min. ET was performed on ice in case of low temperature exposure. Then the cells were washed with PBS. The cells were lysed with reporter lysis buffer (Promega) according to the manufacturer's protocols. Fluorescence intensity of the cell lysate was measured using the microplate reader.

2.7. Confocal laser scanning microscopic observation of the cells after electric treatment

For evaluation of intracellular delivery of ISMB-Luc, 5×10^4 cells were seeded on 0.002% poly-L-lysine coated 35 mm glass bottom dishes. After 18 h, cells were washed with PBS and treated with ET (0.34 mA/cm^2 for 15 min). After ET, the cells were incubated for 1 h at 37 °C in 5% CO_2. After incubation, the cells were observed with a confocal laser scanning microscope A1R+ (Nikon Co. Ltd., Japan). For time-lapse imaging, observation was performed immediately after ET.