GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Hydrogen peroxide enhances the uptake of polyethylenimine/oligonucleotide complexes in A549 cells by activating CaMKII independent of $[Ca^{2+}]_c$ elevation

Z. Ma[1], W.W. Sun[1] and X. Wang[2]

[1]Department of Respiratory Medicine,
The General Hospital of Shenyang Military Area Command,
Shenyang, Liaoning, China
[2]Department of Experimental Medicine,
General Hospital of Shenyang Military Area Command, Shenyang,
Liaoning, China

Corresponding author: Z. Ma
E-mail: zhuangmacn@126.com

Genet. Mol. Res. 13 (2): 2914-2921 (2014)
Received May 13, 2013
Accepted September 13, 2013
Published February 21, 2014
DOI http://dx.doi.org/10.4238/2014.February.21.15

ABSTRACT. Aerosol oligonucleotide therapy has vast potential in pulmonary system diseases. Reactive oxygen species (ROS) play an important role in complex physiological processes such as cell signaling, apoptosis, etc. Therefore, to determine the mechanism of ROS involvement in polyethylenimine/oligonucleotide (PEI/ON) endocytosis in cells, we measured the fluorescence intensities of fluorescein isothiocyanate-labeled ON complexes with PEI and the changes in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) in A549 cells after hydrogen peroxide (H_2O_2) stimulation. Results showed that improved uptake of PEI/ON complexes was independent of the rise of $[Ca^{2+}]_c$ in A549 cells, including the Ca^{2+} inflow and the release of Ca^{2+} from intracellular stores induced by 500 μM H_2O_2. However, the enhanced uptake efficiency

was almost completely abolished by the calcium/calmodulin-dependent protein kinase II (CaMKII) inhibitor and the microtube depolymerized drug. CaMKII-dependent microtube polymerization may be responsible for the enhanced uptake of PEI/ON complexes in A549 cells under oxidative stress conditions. This study is useful for research aimed at improving aerosol oligonucleotide therapy in pulmonary system diseases.

Key words: Hydrogen peroxide; Oligonucleotides; Transfection; Ca^{2+} concentration

INTRODUCTION

Aerosol oligonucleotide therapy has vast potential in pulmonary system diseases including lung cancer and inflammation (Stankova et al., 2005; Crosby et al., 2007; Moschos et al., 2008). However, aerosol oligonucleotide drugs have not generally been associated with high levels of intercellular delivery efficiency. In order to enhance the delivery efficiency, previous studies have largely concentrated on new non-viral vectors and technical means (Lentz et al., 2006; Bolcato-Bellemin et al., 2007). For example, polyethylenimine (PEI) is a highly efficient vector for delivering oligonucleotides and plasmids *in vitro* and *in vivo* due to its "proton sponge effect" (Boussif et al., 1995). Furthermore, genetic material delivery efficiency is not only related to the vector, but also to cell functions under some conditions. Reactive oxygen species (ROS) play an important role in cell functions. ROS also promote cell proliferation and control protein expressions. ROS are increased in a variety of lung diseases (Wood et al., 2010). Enhanced transfection has been related to the extent of ROS production after stress stimuli (Paula et al., 2011); however, the role of ROS in the uptake of PEI/gene particles in cells remains unknown.

Cytosolic calcium concentration $[Ca^{2+}]_c$ is a universal second messenger that is a key component of myriad processes in all cell types. Spatially and temporally controlled changes in $[Ca^{2+}]_c$ are central to the regulation of several key processes ranging from cytoskeleton contractions, cell division, endocytosis, and sensory signaling (Montell, 2005). Previously, we found that the enhanced uptake of PEI/oligonucleotide (PEI/ON) complexes in A549 cells via Ca^{2+} mobilization from intracellular stores was induced by hypo-osmotic stress (Sun et al., 2010). Furthermore, hydrogen peroxide (H_2O_2)-induced elevations in $[Ca^{2+}]_c$ in alveolar type II epithelial cells (Rice et al., 1992) were also reported. In this study, we detected the action of H_2O_2 in the uptake of PEI/ON complexes into alveolar epithelial cells. Furthermore, the action of $[Ca^{2+}]_c$ in the uptake of PEI/ON complexes after oxidative stress stimulation was examined.

MATERIAL AND METHODS

Reagents

Branched PEI (average molecular weight = 25 kDa) was purchased from Sigma-Aldrich Chemical GmbH (Steinhein, Germany). A PEI stock solution was prepared at a 4.3 mg/mL concentration (0.1 M in nitrogen) in 150 mM NaCl. The solution was neu-

tralized with hydrogen chloride and filtered (0.2 μm pore size). An 18-mer sequence (5'-CCTCTTACCTCAGTTACG-3') was fluorescently tagged [5'-fluorescein isothiocyanate (FITC)-labeled], and was obtained from TaKaRa Biotechnology Co., Ltd. (Dalian, China). The FITC-labeled oligonucleotide was complexed with PEI in 150 mM NaCl by slowly adding the PEI to the oligonucleotide while vigorously vortexing the solution. The solution was then allowed to incubate at room temperature for 15-20 min prior to use. The resulting charge ratio is expressed as PEI nitrogen:oligonucleotide phosphorous (N:P) at a 10:1 N:P ratio. Fluo-3 acetoxymethyl ester (Fluo-3 AM) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The 1,2-bis (2-aminophenoxy)-N,N,N,N-tetraacetic acid tetrakis (acetoxymethyl ester; BAPTA-AM) and ethylene glycol-bis (β-aminoethyl ether)-N,N,N,N-tetraacetic acid (EGTA) were obtained from Sigma-Aldrich. The microtubule depolymerization drug nocodazole and the calmodulin-dependent protein kinase II (CaMKII) inhibitor KN93 were obtained from Sigma-Aldrich. The other agents were of analytical grade.

Cell culture

Human lung carcinoma A549 cells (epithelial cell line) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM-glutamine, 56 U/mL penicillin-G, and 56 μg/mL streptomycin sulfate. The cells were regulated at an initial density of 50,000, and were then seeded on 24-well dishes (Costar; Corning, NY, USA) and incubated for 18-20 h before transfection. To determine the effect of H_2O_2 on the transfection, A549 cells were exposed to 500 μM H_2O_2 with Ca^{2+} or without Ca^{2+} isotonic (ISO) medium for 20 min. Cells were then washed with phosphate-buffered saline (PBS) before culturing with the PEI/ON complex medium.

Cells were bathed in ISO medium containing 140 mM NaCl, 5.4 mM KCl, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 3.3 mM $NaHCO_3$, 2.0 mM $CaCl_2$, 10 mM HEPES, 5.5 mM glucose (pH 7.4, adjusted with NaOH, having an osmolarity of 300 mOsm/L). For experiments in the absence of Ca^{2+}, the medium was prepared by substituting the $CaCl_2$ with the same concentration of $MgCl_2$, with the addition of 1 mM EGTA to chelate any trace Ca^{2+}.

Measurement of $[Ca^{2+}]_c$

A549 cells were incubated on cover slips with 3 μM calcium-sensitive probe, Fluo-3 AM, for 40 min at 37°C in ISO medium. Observations were performed with a fluorescence microscope with a 100-W mercury lamp. A shutter was equipped behind the mercury lamp to control the exposure time and the interval time for fluorescence detection. Fluo-3-incubated cells were excited by a mercury lamp with a 488-nm excitation filter, and fluorescence was collected by an objective (40X, oil) with a 510-emission filter. Images were acquired using a cooled CCD camera. The obtained images were quantitatively analyzed for changes of fluorescence intensities within the region of interest (ROI) using the Image J software (National Institutes of Health). The $[Ca^{2+}]_c$ change was represented by the relative fluorescence intensity (F/F_0, intensity after stimulation/basal intensity before stimulation). At least 30 individual cells were selected from three independent experiments, with one characteristic calcium trace plotted to represent >10 similar traces.

Evaluation of the intracellular ON content

Bright-field illumination and fluorescence microscopy were performed with a fluorescence microscope (Olympus IMT-2). The content of ONs in A549 cells was assessed though the fluorescence intensity of FITC in cells. The cells were exposed to 1 μM FITC-labeled ONs, which were complexed with 10 equivalents PEI for 1 h. Then, the cells were washed twice with PBS, fixed in 4% ice-cold paraformaldehyde for 30 min, and the samples were washed twice in PBS. FITC-labeled ONs in cells were imaged with the excitation wavelength (λex) and the emission wavelength (λem) set at 488 and 510 nm, respectively. For quantitation, the area of the cell was selected, and image quantitation was performed using the ImageJ software package. The background fluorescence was subtracted, and the mean fluorescence intensity of the images was determined.

Statistical analysis

All data are reported as means ± standard errors. Statistical comparisons between groups were carried out using the Student *t*-test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Enhanced uptake of PEI/ONs in A549 cells is independent of the $[Ca^{2+}]_c$ elevation induced by H_2O_2

Effects of H_2O_2 on the uptake of PEI/ONs in A549 cells were first examined. Results showed that exposure to 500 μM H_2O_2 for 20 min induced a substantial uptake of PEI/ONs complexes in A549 cells, which was significantly higher than that of the control (Figure 1A, b). Figure 1B shows the significant elevation of the uptake of PEI/ONs induced by H_2O_2 ($P < 0.01$). These results indicated that ROS stimuli could increase the endocytosis of PEI/ON complexes.

In many cells, endocytotic membrane retrieval is accelerated by Ca^{2+} (MacDonald et al., 2005; Wu et al., 2009). In order to clarify the mechanism of enhanced transfection, the change of $[Ca^{2+}]_c$ was detected in our experiments. Results showed $[Ca^{2+}]_c$ elevation induced by 500 μM H_2O_2 in A549 cells (Figure 2A, a, b, and 2B). Next, the same experiments were carried out without Ca^{2+} in the medium. Results showed that the peak of $[Ca^{2+}]_c$ elevation was significantly attenuated ($P < 0.05$), indicating that Ca^{2+} inflow from some channels and Ca^{2+} release from intracellular stores were induced by H_2O_2 in A549 cells (Figure 2A, b, c, and 2B). However, the enhanced endocytosis of PEI/ONs was not at all inhibited without Ca^{2+} in the medium (Figure 1A, c, and 1B; $P < 0.01$). These results indicated that the enhanced endocytosis of PEI/ONs induced by H_2O_2 was not dependent on Ca^{2+} entry. In further experiments, BAPTA-AM, a selective chelator of intracellular Ca^{2+}, was used. The results showed that $[Ca^{2+}]_c$ elevation was exclusively blocked by BAPTA during H_2O_2 stimulation (Figure 2A, d, and 2B). However, BAPTA did not also inhibit the enhanced endocytosis of PEI/ONs induced by H_2O_2 (Figure 1A, d, and 1B). These results indicated that the enhanced uptake of PEI/ONs in A549 cells induced by H_2O_2 was independent of both the Ca^{2+} inflow and the release of Ca^{2+} from intracellular stores.

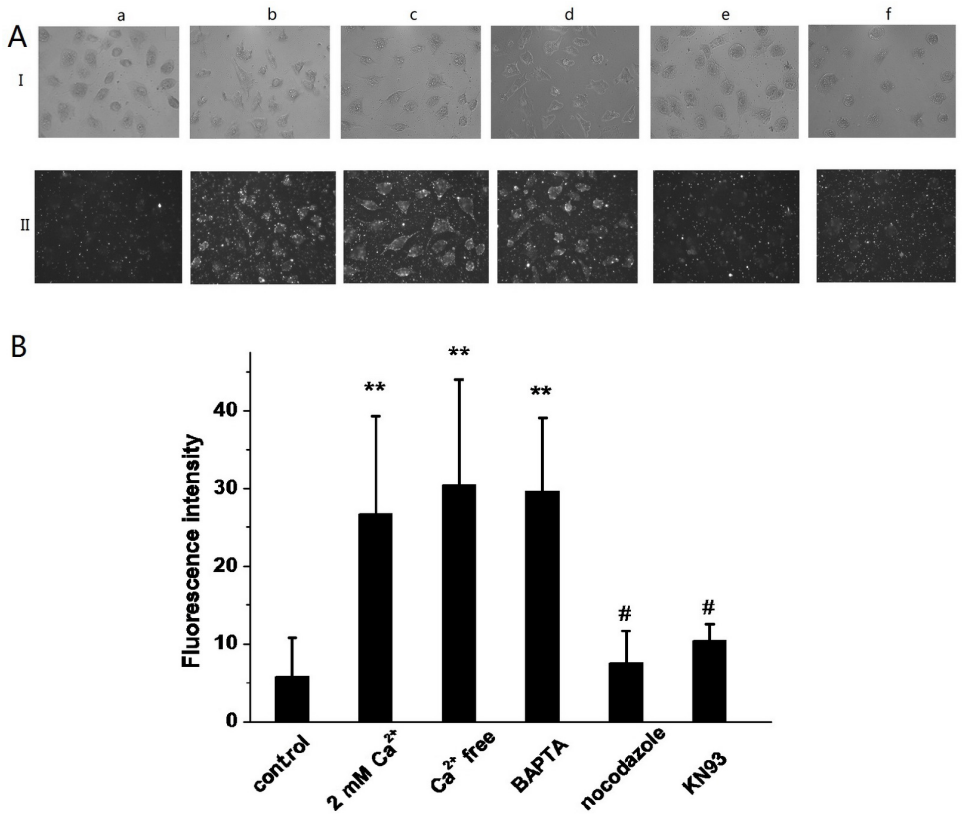

Figure 1. Effect of H_2O_2 on the uptake of FITC-labeled PEI/ONs in A549 cells. **A.** Fluorescence images of A549 cells transfected with FITC-labeled PEI/ONs for 1 h. I) A549 cells were imaged in bright field. II) FITC-fluorescence images were shown with the fluorescence intensity representing the ON concentration. **a**) control; **b**) 500 μM H_2O_2 with 2 mM Ca^{2+} in medium; **c**) 500 μM H_2O_2 and Ca^{2+} free in medium; **d**) 500 μM H_2O_2 and BAPTA [pre-treatment with BAPTA-AM (25 μM) for 30 min]; **e**) 500 μM H_2O_2 and 10 μM nocodazole, pre-treatment for 60 min; **f**) 500 μM H_2O_2 and 2 μM KN93, pre-treatment for 30 min. **B.** Statistical analysis of fluorescence intensity of PEI/ONs in A549 cells. Data are reported as means ± standard error of at least 30 individual cells from three independent experiments. $^{**}P < 0.01$, H_2O_2 increased the uptake of PEI/ONs compared with control. $^{\#}P < 0.01$, nocodazole (10 μM) or KN93 (2 μM) inhibited the uptake of PEI/ONs stimulated by H_2O_2.

CaMKII inhibitor and microtube depolymerization drugs blocked the enhanced uptake of PEI/ONs in A549 cells

The actin cytoskeleton has long been considered to play a role in endocytosis (Jeng and Welch, 2001). In order to study the course, 60 min pre-treatment of 10 μM nocodazole, a microtubule depolymerization drug, was used in the experiment. The results showed that nocodazole significantly blocked the enhanced uptake of PEI/ON complexes induced by H_2O_2 (Figure 1A, e, and 1B). In addition, Ca/calmodulin was previously suggested to play a role during endocytosis (Yao and Sakaba, 2012). In order to further examine this role, 30-min pre-treatment of 2 μM KN93, a CaMKII inhibitor, was used in the experiment. The results (Figure

1A, g, and 1B) showed that KN93 inhibited the enhanced uptake of PEI/ON complexes in A549 cells. These results indicated that the enhanced uptake of PEI/ON complexes in A549 cells was dependent on CaMKII during H_2O_2 stimulation.

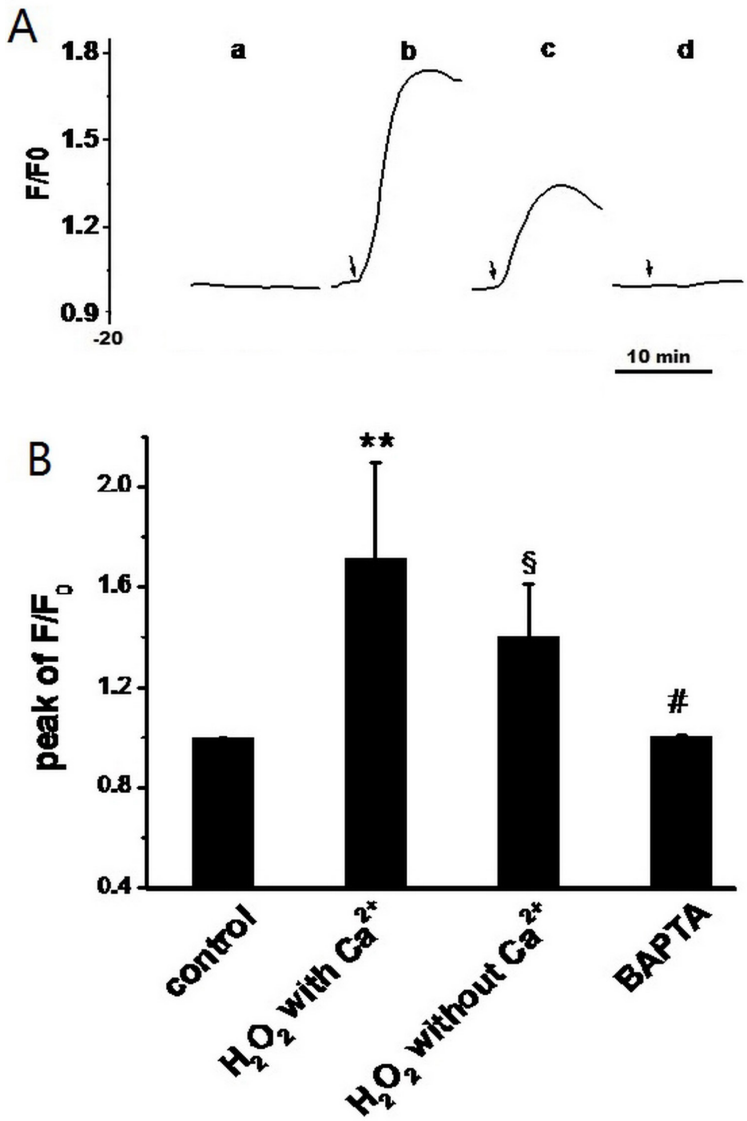

Figure 2. Effect of H_2O_2 on the changes of $[Ca^{2+}]_c$ in A549 cells. **A. a**) control: without change of $[Ca^{2+}]_c$ in ISO; **b**) 500 μM H_2O_2 with Ca^{2+} in medium; **c**) 500 μM H_2O_2 and Ca^{2+} free in medium; **d**) pretreatment with BAPTA-AM (25 μM) for 30 min. **B.** Statistical analysis of peak of $[Ca^{2+}]_c$ in multiple experiments. Data are reported as means ± standard error of at least 10 individual cells from three independent experiments. **$P < 0.01$, 500 μM H_2O_2 (2 mM or without Ca^{2+}) increased the peak of the $[Ca^{2+}]_c$ elevation compared with control. §$P < 0.05$, H_2O_2 decreased the peak of $[Ca^{2+}]_c$ elevation without Ca^{2+} in medium compared with 2 mM Ca^{2+} in medium. #$P < 0.01$, BAPTA-AM (25 μM) completely blocked the $[Ca^{2+}]_c$ elevation induced by H_2O_2 in the presence or absent of extracellular Ca^{2+}.

DISCUSSION

Delivering ONs to the lungs using polymer carriers represents a novel therapeutic approach to the treatment of inflammatory lung diseases (Kim et al., 2012) such as asthma. Paula et al. (2011) reported that the enhanced efficiency of non-viral vector transfection was relative to ROS production using a therapeutic ultrasound. However, the role of ROS in the uptake of PEI/gene particles in cells remains unknown. Having a large surface that is constantly in contact with air oxygen and pollutants makes the lungs an obvious site of major ROS production (Tkaczyk and Vízek, 2007). For this reason, we investigated the effect of ROS on the uptake of PEI/gene particles in cells during ROS stress.

The Ca^{2+} signal is known to be an important second messenger that participates in the process of cytoskeleton contractions and transport (Jena et al., 1997; Sanabria et al., 2009; Wei et al., 2009). Exposure to H_2O_2 was reported to cause elevations in $[Ca^{2+}]_c$ in rat alveolar type II epithelial cells (Rice et al., 1992). This result was confirmed in our experiment in human alveolar type II epithelial cells. Previously, we reported that hyposmotic stress enhanced the uptake of PEI/ON complexes in A549 cells via Ca^{2+} mobilization from intracellular stores (Sun et al., 2010). Thus, we investigated whether the change of $[Ca^{2+}]_c$ might be involved in the enhanced uptake of PEI/ON complexes induced by H_2O_2. H_2O_2-induced $[Ca^{2+}]_c$ elevation was shown to occur via activation of the Ca^{2+}-permeable cation channel in human beta cells (Bari et al., 2009). On the other hand, it was also reported that H_2O_2-induced $[Ca^{2+}]_c$ elevation occurred via the release of Ca^{2+} from the endoplasmic reticulum in the cell (Volk et al., 1997). Our experiment showed that the $[Ca^{2+}]_c$ elevation resulted from both Ca^{2+} inflow and Ca^{2+} mobilization from intracellular stores during H_2O_2 stimulation. However, the enhanced uptake of H_2O_2-induced PEI/ON complexes was not inhibited by eliminating the Ca^{2+} inflow and Ca^{2+} mobilization from intracellular stores during H_2O_2 stimulation. These results indicated that the enhanced uptake of PEI/ON complexes was independent of $[Ca^{2+}]_c$ elevation during H_2O_2 stimulation.

Based on our previous results, we hypothesized that other mechanisms might be involved in this process. It was previously reported that the intracellular transport of therapeutic DNA carriers was not slow relative to random diffusion, whereas a PEI-DNA nanocomposite was shown to involve motor protein-driven transport, which could be inhibited by microtubule depolymerization (Suh et al., 2003). Our results showed that microtubule depolymerization drugs could inhibit the enhanced uptake of PEI/ON complexes, which suggested that the enhanced uptake of PEI/ON complexes occurred via motor protein-driven transport. Furthermore, CaMKII could regulate the actin assembly and structure. Erickson et al. (2008) showed that ROS induced CaMKII activity via a non-Ca^{2+} pathway. In view of the above, we hypothesized that H_2O_2 induced the enhanced uptake of PEI/ON complexes in A549 cells via CaMKII, which was also immediately activated by H_2O_2. The CaMKII blocker inhibited the enhanced uptake of PEI/ON complexes in A549 cells under H_2O_2 stimulation. The results of these experiments suggested that the enhanced uptake of PEI/ON complexes in A549 cells induced by ROS occurred through the activation of CaMKII independent of $[Ca^{2+}]_c$ elevation.

This study provided strong evidence that the enhanced transfection of PEI/ONs into A549 cells occurs in a $[Ca^{2+}]_c$ elevation-independent manner during oxidative stress. CaMKII contributes to increasing PEI/ON endocytosis via cytoskeleton contractions and transportation under oxidative stress conditions. This study may be useful for research aiming to improving aerosol ON therapy in pulmonary system diseases.

ACKNOWLEDGMENTS

Research supported by the Natural Sciences Foundation of Liaoning Province (#2001101031) and the China Postdoctoral Science Foundation Project (#20080441338).

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