Pharm Res (2014) 31:2354–2366
DOI 10.1007/s11095-014-1332-4

RESEARCH PAPER

Ultrasound Enhanced PEI-Mediated Gene Delivery Through Increasing the Intracellular Calcium Level and PKC-δ Protein Expression

Jyun-Lin Lee • Chia-Wen Lo • Claude Inserra • Jean-Christophe Béra • Wen-Shiang Chen

Received: 16 October 2013 / Accepted: 8 February 2014 / Published online: 13 March 2014

ABSTRACT

Purpose Polyethylenimine (PEI), a cationic polymer, has been shown to aggregate plasmid DNA and facilitate its internalization. It has also been shown that combining ultrasound (US) with PEI could enhance and prolong *in vitro* and *in vivo* transgene expression. However, the role US in the enhancement of PEI uptake is poorly understood. This study investigates the impact of US on PEI-mediated gene transfection.

Methods Specific endocytosis pathway siRNA, including clathrin HC siRNA, caveolin-1 siRNA and protein kinase C-delta (PKC-δ) siRNA, are used to block the corresponding endocytosis pathways prior to the transfection of luciferase DNA/PEI polyplexes to cultured cells by 1-MHz pulsed US with ultrasound contrast agent SonoVue®.

Results Transgene expression was found not to be enhanced by US treatment in the presence of the PKC-δ siRNA. We further demonstrated that PKC-δ protein could be enhanced at 6 h after US exposure. Moreover, intracellular calcium levels were found to be significantly increased at 3 h after US exposure, while transgene expressions were significantly reduced in the presence of calcium channel blockers both *in vitro* and *in vivo*.

Conclusions Our results suggest that US enhanced PEI-mediated gene transfection specifically by increasing PKC-δ related fluid phase endocytosis, which was induced by increasing the intracellular calcium levels.

KEY WORDS calcium · fluid phase endocytosis · PKC-δ · polyethylenimine · ultrasound

INTRODUCTION

Non-viral gene transfer methods such as electroporation, ultrasound (US), cationic polymer or cationic lipid, have recently been reported as being potentially applicable to gene therapy (1–6). Gene transfer mediated by non-viral vectors exhibits many of the desired characteristics for future human gene therapy applications, including low immunogenicity, treatment convenience, and site specificity (7–9). US has been shown to enhance the delivery efficiency of drugs or genes into cells (10–14), achieving safe and local delivery of drugs or genes to the target organs by focusing the US beam on the specific target site. The use of skeletal muscle in our *in vivo* study has unique advantages. Muscle constitutes about 30% of adult human body mass and has an abundant blood vascular supply, lending itself to use as target tissue for the production of transgene proteins to act as systemic therapeutic agents (14,15). For example, a US-based systemic gene therapy method allows for the US-mediated delivery of anti-angiogenic genes into skeletal muscles,

Electronic supplementary material The online version of this article (doi:10.1007/s11095-014-1332-4) contains supplementary material, which is available to authorized users.

J.-L. Lee • C.-W. Lo • W.-S. Chen (✉)
Department of Physical Medicine and Rehabilitation
National Taiwan University Hospital
National Taiwan University College of Medicine
Taipei, Taiwan, Republic of China
e-mail: wenshiang@gmail.com

C. Inserra • J.-C. Béra
INSERM, U1032, LabTAU, Université Claude Bernard Lyon 1
151 Cours Albert Thomas, 69424 Lyon, France

W.-S. Chen
Division of Medical Engineering Research
National Health Research Institutes
Miaoli, Taiwan, Republic of China

significantly inhibiting the growth of orthotopic tumors in mice (16). However, the US method suffers from low transfection efficiency and short transgene expression. It has been found that combining US with cationic polymers such as polyethylenimine (PEI) could enhance and prolong the transgene expression either *in vitro* or *in vivo* (17). However, the role of US on the enhancement of PEI uptake is still not well understood.

Most reports on cellular uptake mechanisms suggest that large DNA/PEI polyplexes enter the cells by means of endocytosis. Endocytosis can be classified into a number of distinctly different routes, including pinocytosis which is recognized as being responsible for the uptake of macromolecules, such as PEI. Pinocytosis can be further subdivided into macropinocytosis, clathin-mediated endocytosis, and caveolin-mediated endocytosis (18). Macropinocytosis has been shown to selectively internalize suspended macromolecules (for molecules up to 1 μm in diameter). In contrast, clathrin-mediated endocytosis prefers to internalize macromolecules with diameters of up to 200 nm, while caveolin-mediated endocytosis uptakes smaller molecules (diameter <100 nm) (19,20). Several studies have demonstrated that DNA/PEI polyplexes enter the cells *via* clathrin-mediated endocytosis; but other research has shown that both caveolin and clathrin-mediated endocytosis are involved in the internalization of PEI (21–23). Moreover, additional pathways such as fluid phase endocytosis have also been found to contribute to PEI transfection (24). Given these conflicting results, the exact pathway for internalization of polyplexes is still unknown.

In addition, our understanding is incomplete for the mechanisms for the cellular uptake of smaller molecules, such as genes or drugs, following US exposure. One explanation focuses on the production of transient pores on cell membranes (25,26). However, several recent studies have demonstrated that endocytosis may also be involved in the delivery process following US exposure (27,28). Other studies have shown that the enhancement of endocytosis after US exposure is associated with H_2O_2 formation and a rise in intracellular calcium levels (29,30). The present study aims to determine the role of endocytosis in US-facilitated DNA/PEI polyplex transfection. We first investigated the transfection efficiency and cellular >uptake following pretreatment with endocytosis inhibitors, siRNA, after which the colocalizations of PEI and endocytosis markers were observed using confocal microscopy. The intracellular calcium level was then examined after US exposure. We also evaluated the effect of varying calcium concentrations during PEI-mediated gene transfection. Our findings suggest that US enhances PEI-mediated gene transfection through protein kinase C-delta (PKC-δ) and calcium-associated fluid phase endocytosis.

MATERIALS AND METHODS

Chemicals

PEI (Polyplus Transfection, Illkirch, France), a 22 kDa linear polyethylenimine (PEI), was used according to the manufacturer's protocols. PEI-FluoF (green) and PEI-FluoR (red), fluorescein-conjugated linear polyethylenimine, were all purchased from Polyplus Transfection. Calcium channel blockers, verapamil and nifedipine, were purchased from Sigma-Aldrich (Missouri, USA).

Cell Culture and Expression Vector

C2C12 cells (murine skeletal muscle cells) were purchased from American Type Culture Collection (ATCC, Virginia, USA). These cells were grown in Dulbecco's modified Eagle's medium (Gibco, New York, USA) supplemented with 10% fetal bovine serum (Gibco, New York, USA), 1% mixture of penicillin G, streptomycin and amphotericin B (Gibco, New York, USA) at 37°C in 5% CO_2. Firefly luciferase cDNA (luc) was subcloned into the pCI-neo Vector, resulting in the constructs pCI-neo-luc. Plasmid pCI-neo-luc was transformed into competent Escherichia coli DH5α, and endotoxin-free plasmid DNA was purified using the Qiagen EndoFree Plasmid Max kit (Qiagen, California, USA) according to the manufacturer's instructions.

Ultrasound Apparatus and Gene Transfer

In Vitro Study

C2C12 cells were seeded in 24-well plates at an initial density of 2×10^4 in 0.5 ml of growth medium and incubated for 24 h before US exposure. The 24-well plates were placed above a US probe with a thin layer of US gel in between, and the wells were individually exposed to US by aligning the center of the exposed well with the center of the US probe. The culture medium contained 1 μg of plasmid DNA and PEI at an N/P ratio of 5, *i.e.*, the ratio of nitrogen atoms on PEI to phosphates on DNA is 5 to 1. US was generated by a commercial gene transfection device, Sonitron 2000 (Rich-Mar Co., Oklahoma, USA), equipped with a 1 MHz probe in an average intensity of 2 W/cm^2, and a duty cycle (DC) of 20% for 2 min. The diameter of the US probe was 10 mm. In each well, 500 μL of culture medium containing 5 μL of SonoVue contrast agent (SonoVue® Bracco, Milan, Italy) are prepared for US exposure (31). After 6 h of US exposure, the cells were washed three times with ice-cold phosphate-buffered saline (PBS) and were further incubated with a culture medium at 37°C for 48 h. The cells were stripped from the culture plates and suspended in a luciferase lysis buffer (Promega, Wisconsin, USA). Cell suspensions were mixed and then centrifuged at 12,000×*g* for

10 min at 4°C. The supernatant was assayed with a luciferase assay substrate kit (Promega, Wisconsin, USA) and luciferase activity was measured using a microplate luminometer infinite M200 (Tecan, Groedig, Austria). Luciferase activity was normalized to the protein content of the cells. Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, California, USA).

In Vivo Study

In vivo gene transfection was performed by exposing the injection sites to the US field of the Sonitron 2000 (Rich-Mar Co., Oklahoma, USA) sonoporation device following intramuscular injection of the US contrast agent (SonoVue® Bracco, Milan, Italy) and reporter plasmids. US was generated by a 1 MHz probe of the same gene transfection device with an average intensity of 2 W/cm^2, a DC of 20%, and a pulse repetition frequency of 200 Hz. The diameter of the US probe was 10 mm. The durations of US exposure for *in vitro* and *in vivo* are the optimal parameters we obtained from our previous studies (32,33). The SonoVue® was a lipid-shelled US contrast agent composed of microbubbles filled with sulfur hexafluoride gas (34). The diameters of the microbubbles ranged from 2.5 to 6.0 μm. Upon use, the SonoVue was reconstituted in PBS containing $2\sim5\times10^8$ microbubbles per mL. Mixtures of 10 μg plasmid DNA and SonoVue (30% v/v) with a predetermined amount of PEI (N/P ratios of 0.5) were intramuscularly injected into the thigh muscle of 6–8 week old male BALB/c mice (17,33). The injection site of each mouse then received 5 min of US exposure. At predetermined times following injection, mice were anesthetized by isoflurane and intraperitoneal injected with 100 μl of luciferin (30 mg/ml). The whole body distribution of luciferase expression was monitored by an IVIS imaging system (Xenogen, California, USA). Luciferase activity was quantitatively measured using Live Image 2.5 software (Xenogen, California, USA).

Quantification of DNA/PEI Uptake

To measure DNA uptake, DNA/PEI-FluoF nanoparticles were transfected to C2C12 (2×10^4 cells/0.5 ml) by US and incubated in a 24-well plate for 6 h. Intracellular uptake of DNA/PEI-FluoF was measured by a LSRII flow cytometer (BD Biosciences, California, USA) and analyzed using FlowJo software (Treestar Software, California, USA).

Cell Viability Assay

In vitro cytotoxicity tests were performed using an MTT assay. Cells were seeded in 24-well plates at an initial density of 2×10^4 in 0.5 ml of a growth medium and incubated for 24 h. One day or 2 days after US exposure, the cells were then treated with an MTT reagent (Sigma-Aldrich, Missouri, USA), and were further incubated for 4 h at 37°C. The medium was removed and 1 ml DMSO was added to dissolve the MTT product (formazan crystals). The plate was gently shaken for 15 min to achieve complete dissolution. Aliquots (100 μl) of the resulting solution were transferred to 96-well plates and absorbance was recorded at 570 nm using the microplate spectrophotometer system. Relative cell viability was calculated as $(A_{treat}/A_{control}) \times 100\%$.

Endocytosis Related Protein Depletion Experiments

The three major endocytosis pathways (clathrin, caveolin-mediated or fluid phase endocytosis) were selectively and respectively inhibited using Clathrin heavy chain (HC) siRNA (Santa Cruz Biotechnology, California, USA), Caveolin-1 siRNA (Santa Cruz Biotechnology, California, USA), PKC-δ siRNA (Santa Cruz Biotechnology, California, USA) or control siRNA (Santa Cruz Biotechnology, California, USA). C2C12 cells were seeded in 24-well plates at an initial density of 2×10^4 in 0.5 ml of growth medium and incubated for 24 h. They were then transfected with siRNA duplexes using Lipofectamine RNAi MAX reagent (Invitrogen, California, USA) according to the manufacturer's instructions. The transfection effect and DNA/PEI uptake were analyzed 48 h after the siRNA transfection. The optimal siRNA concentration was determined to be 10 nM (Fig. S1 in Supplementary Material).

Western Blotting and Immunofluorescence

Cells or tissues were lysed in a lysis buffer (25 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, and protease and phosphatase inhibitors). Lysate protein concentration was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, California, USA) and normalized for loading. Proteins were separated by SDS-PAGE using 8 or 12% gels, and the gels were then transferred to a PVDF membrane (Millipore, Massachusetts, USA), which was blocked with 5% BSA in a TBS/TWEEN 20 (TBST) buffer which contains 140 mM Sodium Chloride, 3.0 mM Potassium Chloride, 25 mM Tris and 0.1% Tween 20. (Amresco, Solon, Ohio, USA), and then incubated in 5% BSA/TBST overnight at 4°C with the primary antibody (1:500) against clathrin heavy chain (BD Biosciences, California, USA), primary antibody (1:500) against PKC-δ(Cell Signaling Technology, Massachusetts, USA), primary antibody (1:200) against caveolin-1 (Santa Cruz Biotechnology, California, USA), and primary antibody (1:1,000) against Pan-Actin (Cell Signaling, Massachusetts, USA). Anti-rabbit IgG-HRP or anti-mouse IgG-HRP secondary antibodies (Southern Biotech, Alabama, USA) were used to recognize the primary antibodies. The specific proteins were visualized by a chemiluminescence reagent detection system (Millipore, Massachusetts, USA). Band quantification

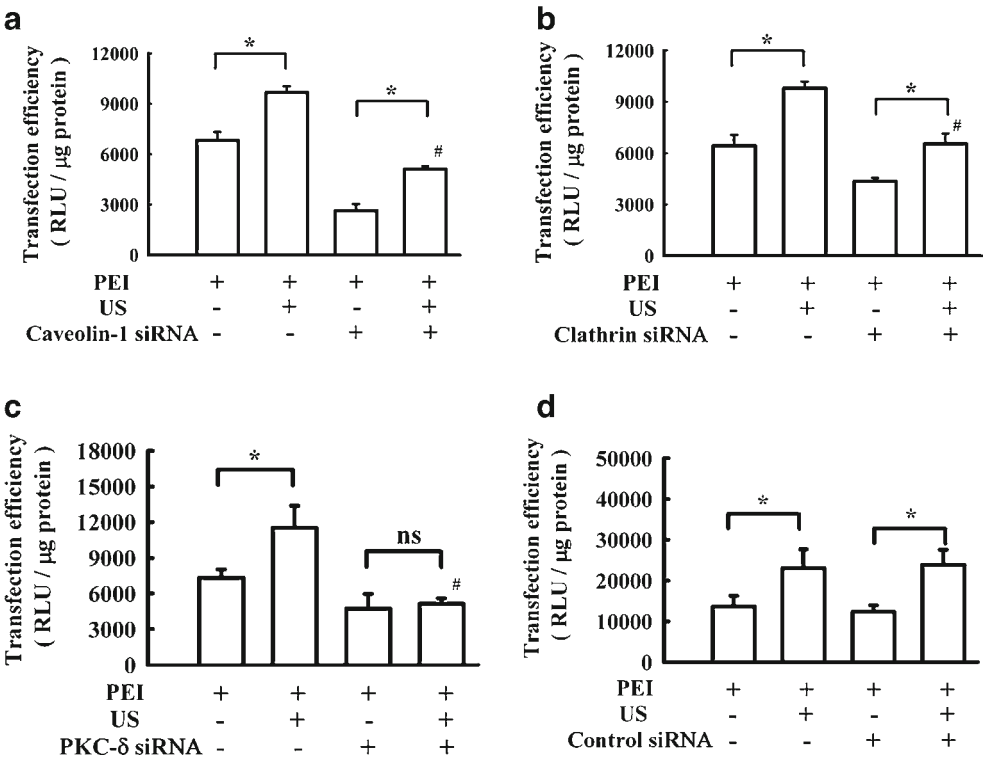

Fig. 1 Effect of endocytosis pathway depletion by siRNA on the transgene expression. C2C12 cells were pre-incubated with 10 nM clathrin (**a**), caveolin-1 (**b**), PKC-δ (**c**) or control (**d**) siRNA for 48 h before transfection with or without US. The transgene expression of DNA/PEI polyplexes were all reduced after depletion. However, US could still enhance the expression level of the **a**, **b** and **d** groups, but not the **c** group. All results are expressed as the mean ± SEM for six independent experiments. $^{*}P < 0.05$; $^{\#}P < 0.05$ *vs.* DNA/PEI with US group; *ns* not significant.

was performed using a BioSpectrum 600 Imaging System (UVP, California, USA).

Determination of Intracellular Calcium Level

Following US exposure at different time points, C2C12 cells were loaded with 3 μM calcium dye Fluo-4 (Molecular Probes, Oregon, USA) for 30 min. Cells were trypsinized and washed with ice-cold PBS (calcium and magnesium free), and then resuspended. The intracellular calcium level was determined by flow cytometry (BD Biosciences, California, USA) and analyzed using FlowJo software (Treestar Software, California, USA).

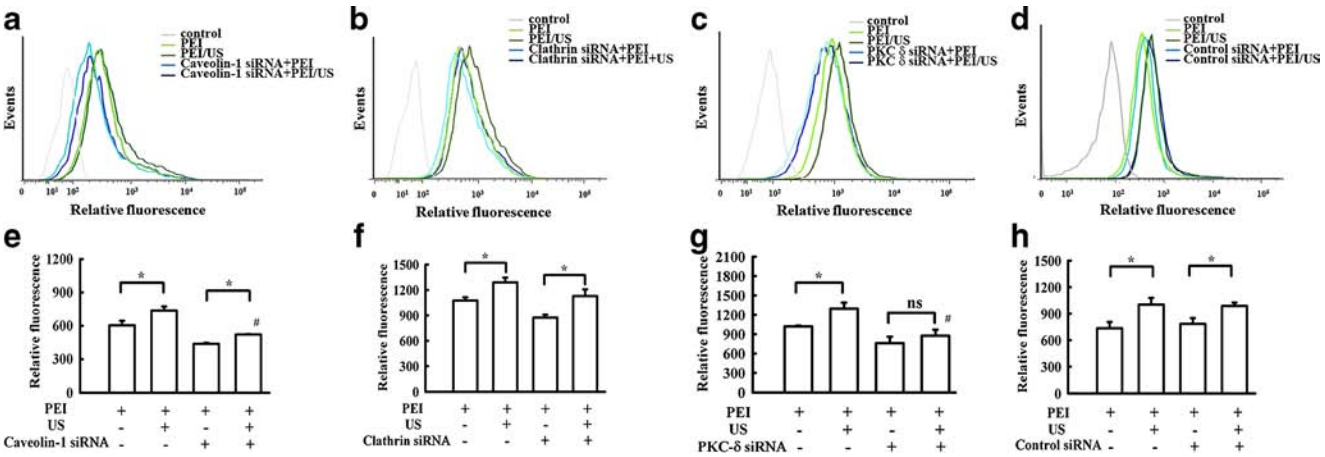

Fig. 2 Effect of endocytosis pathway depletion on the enhancement of PEI-mediated gene transfer by US. Fluorescence labeled PEI was mixed with DNA for the three siRNA depletion conditions, 10 nM clathrin, caveolin-1, PKC-δ siRNA or control siRNA for 48 h (**a**–**d**). The mean intracellular fluorescence intensity was measured by flow cytometry. Although all fluorescence levels were reduced after depletion, they could still be enhanced by US (**e**, **f**, **h**), with the exception of the PKC-δ siRNA group (**g**). The *grey curves* represent the non-stained controls in the histograms. All results are expressed as the mean ± SEM for four independent experiments. $^{*}P < 0.05$; *ns* not significant. $^{\#}P < 0.05$ *vs.* DNA/PEI with US group.

Colocalization Studies

To identify the endocytic pathway involved in the internalization of DNA/PEI-FluoR nanoparticles, we used a colocalization assay in living cells. C2C12 cells were co-incubated with DNA/PEI and different endocytosis markers. A 10 kDa Alexa Fluor 488 conjugate dextran (Molecular probes, Oregon, USA), a fluid-phase endocytosis uptake marker, was used for macropinocytosis; Alexa Fluor 488 conjugate transferrin (Molecular Probes,

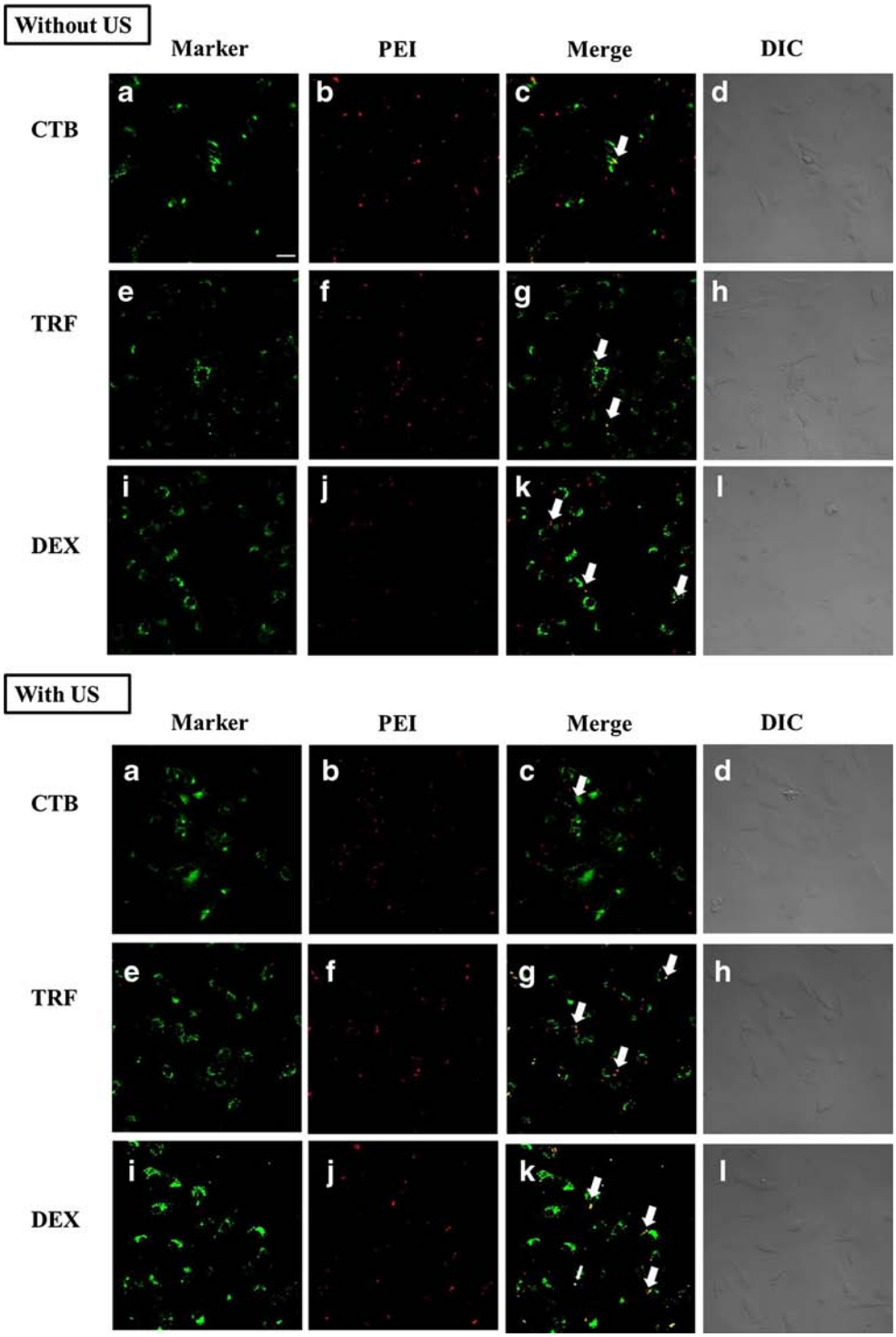

Fig. 3 Colocalization of DNA/PEI polyplexes with the endocytosis pathway markers with or without US exposure. (**a**) Alexa Fluor 488 CTB (*green*) and (**b**) DNA/PEI-FluoR (*red*) polyplexes, (**e**) Alexa Fluor 488 TRF (*green*) and (**f**) DNA/PEI-FluoR (*red*) polyplexes, or (**i**) Alexa Fluor 488 DEX (*green*) and (**j**) DNA/PEI-FluoR (*red*) polyplexes were added to the cultured cells. After 6 h of incubation, living cells were imaged using a confocal laser scanning microscope. Marked colocalization (*yellow*) of DNA/PEI polyplexes was found in the DEX groups only. DIC, differential interference contrast image. *Scale bar* = 10 μm. Similar results were obtained in four independent experiments, a representative one is shown here.

Oregon, USA) was used to determine the clathrin-mediated endocytosis; while Alexa Fluor 488 conjugate Cholera Toxin Subunit β (Molecular Probes, Oregon, USA) was used for caveolin-mediated endocytosis. After 6 h of US exposure, the cells were washed twice with ice-cold PBS and then incubated in fresh medium. The colocalizations of DNA/PEI and the endocytosis marker were observed using a Zeiss LSM 780 laser scanning confocal microscope (Leica TCS SP5 Confocal Spectral Microscope Imaging System, Wetzlar, Germany).

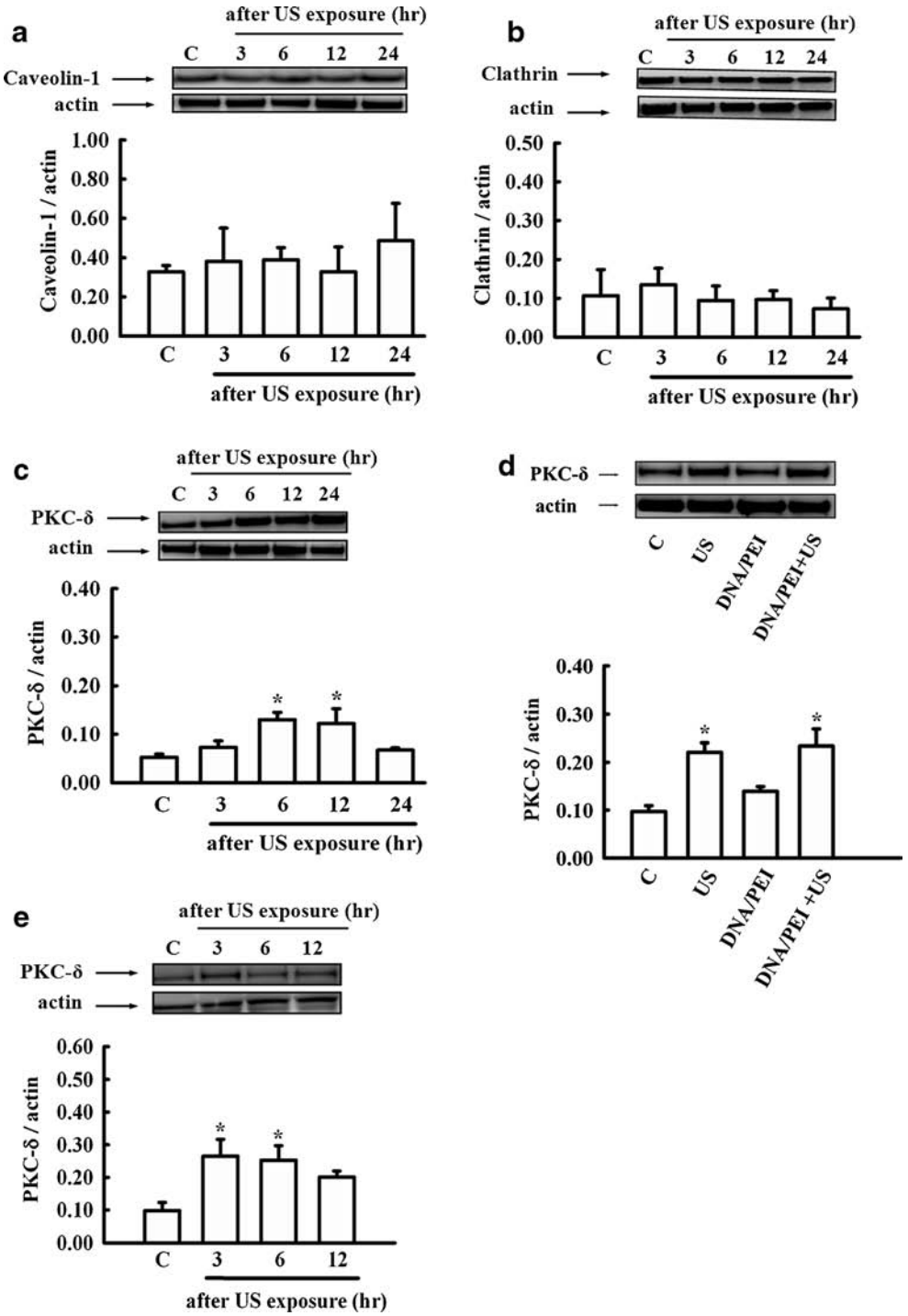

Fig. 4 Effects of US on intracellular caveolin-1, clathrin and PKC-δ protein expression *in vitro* and *in vivo*. **a**–**c** The time course of *in vitro* (**a**) caveolin-1, (**b**) clathrin, (**c**) PKC-δ proteins 3–24 h after US exposure. (**d**) Comparison of PKC-δ protein expression at 6 h in conditions with or without the addition of DNA/PEI and with or without US exposure. (**e**) *In vivo* PKC-δ protein expression at 3–12 h after US exposure. PKC-δ protein levels were significantly increased after US exposure. *C* is control group without US exposure or the addition of DNA/PEI polyplexes. All results are expressed as the mean ± SEM for four independent experiments. $^{*}P < 0.05$ *vs.* *C*.

Statistical Analysis

All data are presented as the mean ± SEM. The differences were analyzed using one-way ANOVA, with "p" <0.05 considered statistically significant.

RESULTS

Effect of Endocytosis Pathway Depletion on the Enhancement of PEI-Mediated Transgene Expression by US

To investigate the role of US on the different internalization pathways for PEI-mediated gene delivery, C2C12 cells were pre-incubated with 10 nM clathrin, caveolin-1, PKC-δ siRNA or control siRNA for 48 h before DNA/PEI polyplexes transfection. Luciferase DNA/PEI polyplexes were then added to the medium of the cultured cells and treated with US. The transfection efficiency was determined by luciferase activity assay after 24 h. As shown in the first two columns of Fig. 1a–d, US shows its ability in enhancing the transgene expression as expected. Pre-incubated cells with clathrin, caveolin-1 or PKC-δ siRNA all exhibited reduced expression levels of transfected DNA. However, following the depletion of clathrin or caveolin-1 pathway, US could still enhance its transgene expression (Fig. 1a and b). In contrast, the transgene expression level was not significantly affected by US after depleting the PKC-δ pathway (Fig. 1c), implying an association between PKC-δ pathway and the US-facilitated transgene expression.

Effect of Endocytosis Pathway Depletion on the Enhancement of PEI-Mediated Gene Transfer by US

To further determine whether the reduction of transgene expression was due to the decreased DNA/PEI uptake, fluorescence-labeled DNA/PEI-FluoF complexes were transfected into cells after being pre-incubated with 10 nM clathrin, caveolin-1 or PKC-δ siRNA for 48 h. Uptake efficiency was determined by measuring the relative intensities of fluorescence unit (RFU) at 6 h after US exposure. As shown in Fig. 2, more DNA/PEI polyplexes were internalized in the US exposure groups after the pretreatment of cells with caveolin-1 (438.0±8.5 *vs.* 536±15.5 RFU) or clathrin siRNA (872.7±34.9 *vs.* 1127.3±79.4 RFU), but not the PKC-δ siRNA (760.3±99.6 *vs.* 877.3±79.4 RFU).

Colocalization of DNA/PEI Polyplexes with Endocytosis Pathway Markers

We used three different endocytosis pathway markers to characterize the role of US on the endocytosis of DNA/PEI

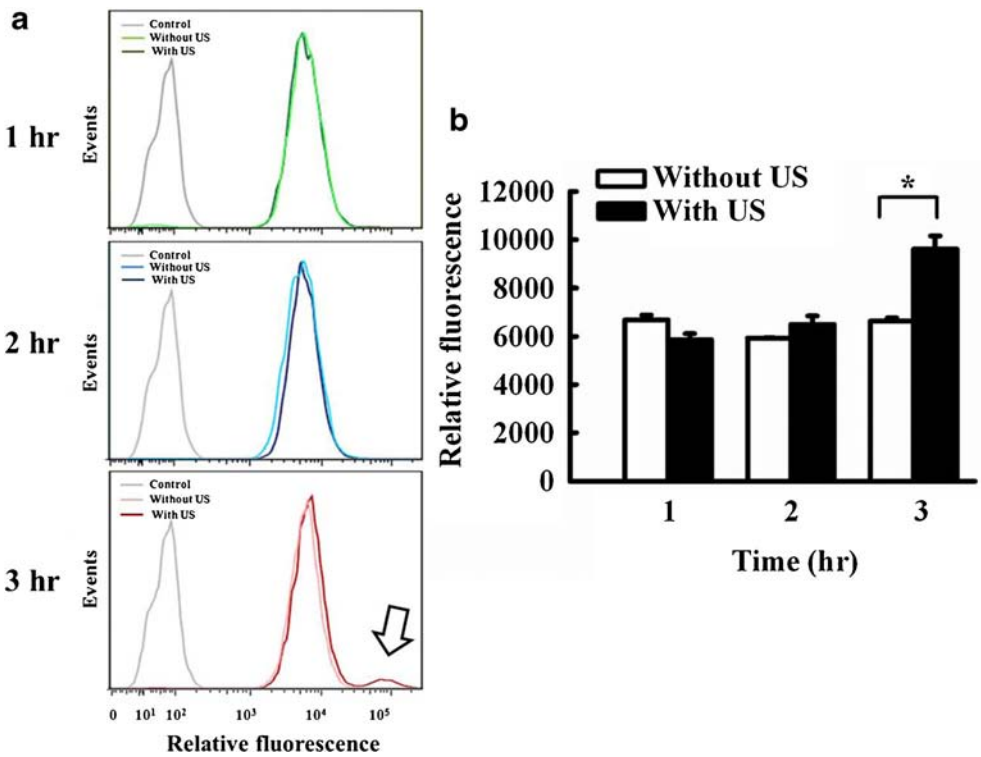

Fig. 5 US increases the intracellular calcium level. Cells were loaded with 3 μM calcium dye Fluo-4 for 30 min at the time points of 1, 2 and 3 h with or without US exposure, and the intracellular calcium levels were determined by flow cytometry (**a**). Quantitative assessment of the intracellular mean fluorescence intensities showed increased calcium levels at 3 h in the US group. The *grey curves* (**a**–**d**) represent the non-stained controls in the histograms. All results are expressed as mean ± SEM for four independent experiments. *P < 0.05.

polyplexes. The fluorescein-conjugated linear PEI (PEI-FluoR) was incubated in the presence of Alexa Fluor 488 Cholera Toxin Subunit β (CTB), transferrin (TRF) or dextran (DEX). The colocalizations of DNA/PEI polyplexes and endocytosis markers were observed using a confocal microscope 6 h after transfection. As shown in Fig. 3, although DNA/PEI polyplexes were found to colocalize with CTB (Fig. 3c), TRF (Fig. 3g) or DEX (Fig. 3k), significantly more colocalization of DNA/PEI polyplexes were found in DEX groups. This result suggests that fluid phase endocytosis was the major route for the uptake of DNA/PEI polyplexes.

Effects of US on Endocytosis-Associated Protein Expression

To explore the effects of US on the endocytosis-associated protein expression, the three major endocytosis-associated proteins (clathrin, caveolin-1 and PKC-δ) were analyzed using the western blot 3–24 h after US exposure after US exposure (Fig. 4a–c, no DNA/PEI). Compared with control cells, similar levels of caveolin-1 or clathrin protein expression were observed after US exposure at time points ranging from 3 to 24 h (Fig. 4a and b). However, as shown in Fig. 4c, PKC-δ protein expressions in C2C12 cells were significantly increased from 6 to 12 h after US exposure. Similar trends were found after the addition of DNA/PEI polyplexes. PKC-δ protein expression was significantly increased at 6 h after US exposure (Fig. 4d) in the *in vitro* condition. *In vivo* study also shows the enhancement of PKC-δ protein expressions from 3 to 6 h after US exposure (Fig. 4e).

Effects of US on Intracellular Calcium Levels

To investigate the effects of US on intracellular calcium levels, cultured cells were loaded with 3 μM calcium dye Fluo-4 for 30 min at three time points: 1, 2 and 3 h after US exposure. The intracellular calcium levels were then determined by flow cytometry. As shown in Fig. 5, no significant difference in mean fluorescence intensity of calcium dye Fluo-4 was found in the first 2 h regardless US exposure. However, representative histogram shows significant increase in the intracellular $Ca2^{+}$ content in a small subgroup of C2C12 cells of the US group at 3 h (Fig. 5b).

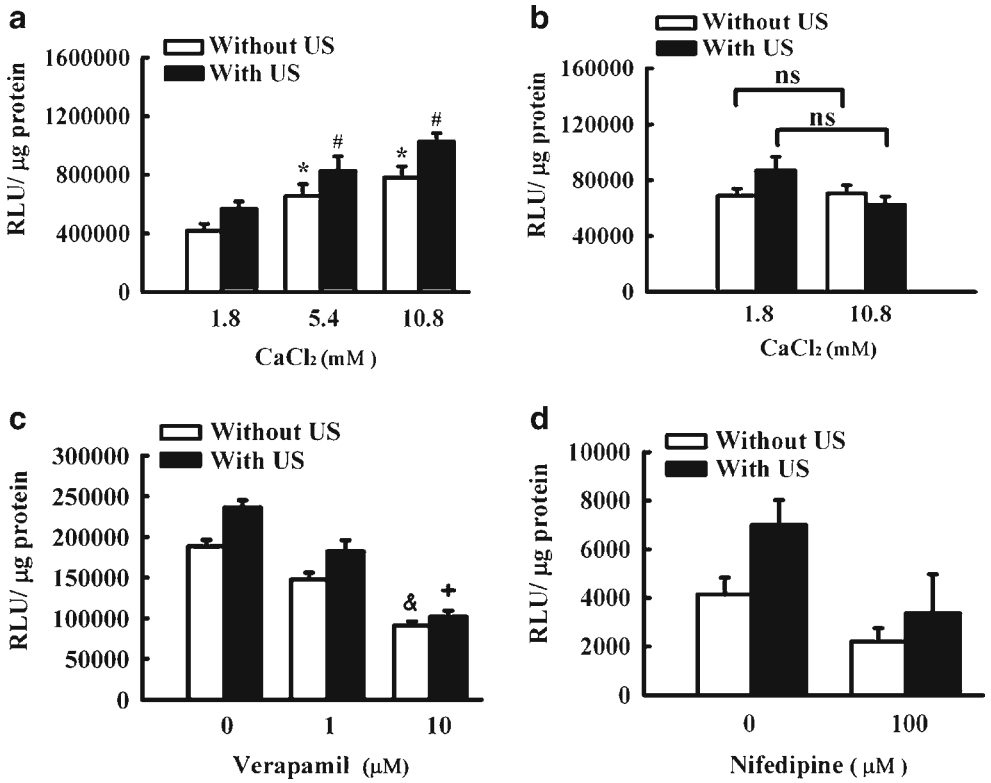

Fig. 6 Effect of calcium on PEI-mediated gene transfer *in vitro*. (**a**) C2C12 cells were co-incubated with 1.8, 5.4 or 10.8 mM of $CaCl_2$ and DNA/PEI polyplexes. The transgene expressions increase with $CaCl_2$ concentration; (**b**) C2C12 cells were co-incubated with DNA/lipofetamine 2000 lipoplex at two different $CaCl_2$ concentration levels, 1.8 or 10.8 mM. Calcium level showed no effect on gene transfer by lipofetamine; (**c**, **d**) C2C12 cells were pre-incubated with a calcium channel blocker, verapamil (**c**) or nifedipine (**d**) for 1 h, and were then co-incubated with the DNA/PEI polyplexes for 6 h. The transgene expressions were determined by luciferase assay 48 h after transfection. Reduced transfection levels were found in the presence of calcium channel blockers. All results are expressed as the mean ± SEM for four independent experiments. *, $P < 0.05$ *vs.* with 1.8 mM of $CaCl_2$ and without US group. #, $P < 0.05$ *vs.* with 1.8 mM of $CaCl_2$ and with US group. &, $P < 0.05$ *vs.* without verapamil and without US group. $^{+}$, $P < 0.05$ *vs.* without verapamil and with US group. *ns* not significant.

Influence of Calcium and Calcium Channel Blockers on the US Enhanced PEI-Mediated Gene Transfer

To further determine the roles of US and calcium on the PEI-mediated gene transfection, we increased the $CaCl_2$ concentrations in the culture medium from 1.8 mM to 10.8 mM during the gene transfer. Elevated calcium concentrations had no significant effect on cell cytotoxicity (Fig. S3 in Supplementary Material). As shown in Fig. 6a, the luciferase expressions were enhanced dose-dependently by increasing the concentrations of $CaCl_2$ in the culture medium. Moreover, combining PEI and US also provided better transfection efficiency after raising calcium levels. Lipofetamine 2000, a widely used non-viral gene transfer reagent, can be used for gene delivery *via* the endocytosis pathway. However, our study showed that increasing the calcium levels to 10.8 mM had no significant effect on transgene expression as compared with the control (1.8 mM of calcium level) when lipofetamine 2000 was employed.

We also used calcium channel blockers (verapamil or nifedipine) to determine whether calcium contributed to US-enhanced PEI-mediated transfection. Cells treated with verapamil or nifedipine were incubated with DNA/PEI polyplexes for 6 h and then washed three times with ice-cold PBS. The cells were further incubated with culture medium at 37°C for 48 h, and the transfection efficiency was determined using the luciferase assay. As shown in Fig. 6c and d, in the presence of verapamil at 10 μM or nifedipine at 100 μM, the transfection efficiency was significantly reduced after gene transfer using PEI with or without US exposure. This result suggests that calcium could affect PEI-mediated gene delivery.

Verapamil Inhibits *In Vivo* US-Induced Gene Transfer

To evaluate the effect of the calcium channel blocker, verapamil, on US-enhanced *in vivo* PEI/DNE polyplex transfection, we injected verapamil (15 mg/kg) into the mouse thigh muscle one hr before performing the US-enhanced DNA/PEI polyplex transfection. The transfection consisted of injecting a mixture of 10 μg DNA, PEI (N/P ratio 0.5), and 30% v/v SonoVue before exposure to 1 MHz, 2 W/cm^2 US for 5 min. The *in vivo* luciferase activity was measured by an IVIS system 3 days after US exposure. As shown in Fig. 7, the level of luciferase activity was significantly decreased by verapamil to a level about 20% of that found in the control group (US without verapamil).

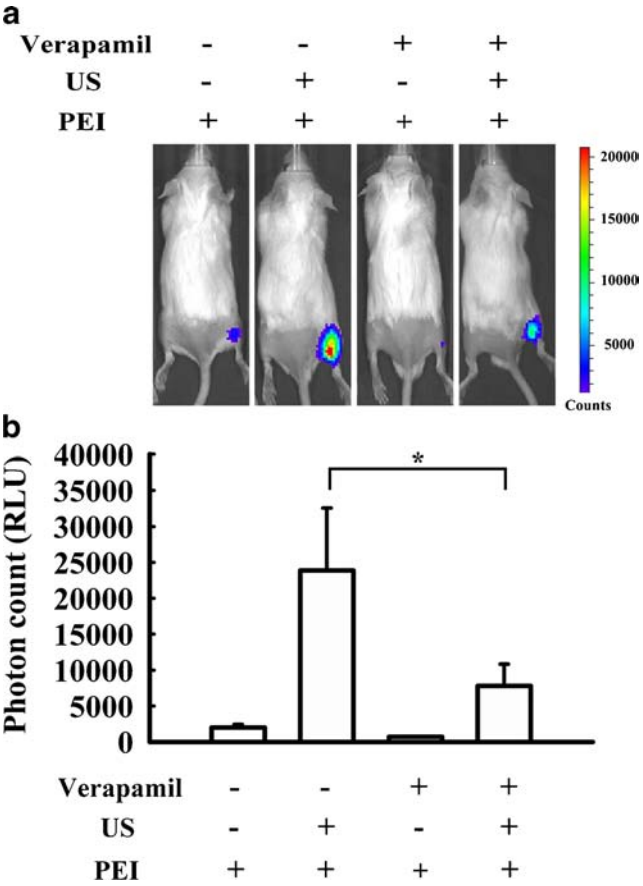

Fig. 7 Effect of verapamil on the transfection of plasmid DNA *in vivo*. DNA (10 μg) was gently mixed with PEI at an N/P ratio of 0.5 before being injected into the thigh muscle of Balb/C mice and transfected by sonoporation. The thigh muscle had been pre-treated with verapamil (15 mg/kg) 1 h before. Luciferase activity in the target tissue was measured by an IVIS system 3 days after US exposure. Photon count (RLU) represents the luciferase activity of the transgene expression. Luciferase activity in the verapamil group (**b**) was significantly lower. All results are expressed as mean ± SEM for eight independent experiments. *, $P < 0.05$ *vs.* without verapamil group.

DISCUSSION

Previous research on the pathway of DNA/PEI polyplex transfection is characterized by conflicting results. Our results shown in Figs. 1 and 2 demonstrate that transfection efficiency or cellular uptake of DNA/PEI polyplex was significantly reduced by using the three siRNA. However, in contrast with pretreatment of clathrin or caveolin-1siRNA, US did not enhance the transfection or cellular uptake of PEI following the pretreatment of cultured muscle cells with PKC-δ siRNA (Figs. 1 and 2). In addition, much more colocalization of the DNA/PEI polyplexes with the fluid phase endocytosis markers, dextran, was found (Fig. 3). Moreover, we found that PKC-δ protein expressions were significantly increased 6–12 h after US exposure *in vitro* (Fig. 4c) or 3–6 h after US exposure *in vivo* (Fig. 4e). Nonetheless the clathrin and caveolin-1 protein expressions could not be changed by US (Fig. 4b and c). These results suggest that gene transfer by DNA/PEI polyplexes might be routed through the clathrin, caveolin, and fluid phase endocytosis, whereas US enhances PEI-mediated gene transfection specifically by increasing the PKC-δ protein expression.

Endocytosis blockers have been used in exploring the pathways of PEI-mediated gene transfer or drug delivery. For example, Gersdorff *et al.* (21) provide indirect evidence of endocytosis' involvement in PEI polyplexes uptake by inhibitors of the clathirn-mediated endocytosis, while other studies show that rottlerin, an inhibitor of fluid phase endocytosis, can be used to specifically decrease cellular uptake of PEI (24). Although endocytosis blockers have been widely used to explore endocytosis pathways, they have poor specificity in inhibiting distinct endocytosis pathways (35). Hence, in this study, we chose the specific endocytosis pathway siRNA, clathrin HC, caveolin-1 and PKC-δ to specifically block the corresponding endocytosis pathways. Figures 1 and 2 show that the transgene expression level was not significantly affected by US after depleting the PKC-δ pathway by the corresponding siRNA (Figs. 1c and 2g), implying an association between PKC-δ pathway and the US-facilitated DNA/PEI polyplexes delivery.

Another important question to be answered is how US enhances the fluid phase endocytosis. Previous research shows that rottlerin, a selective inhibitor of PKC-δ and the fluid phase endocytosis, suppresses PEI-mediated gene transfection (24,36,37). As shown in Fig. 4c and e, PKC-δ protein expressions were significantly increased at 6 to 12 h after US exposure (*in vitro*) and at 3 to 6 h after US exposure (*in vivo*). The possible relevance of US and PKC-δ includes apoptosis and calcium. It is known that PKC-δ is activated during apoptosis, following proteolytic cleavage of caspase (38). It has also been demonstrated that US induced the apoptosis *in vitro* (39). However, in our experiments, there is no significant cytotoxic effect on cells by US exposure (Fig. S2 in the Supplementary Material). Moreover, PKC is a family of serine- and threonine-specific protein kinases that can be activated by calcium. US exposure has been reported to increase intracellular calcium levels (40). The present study also found that intracellular calcium levels significantly increased 3 h after US exposure (Fig. 5). Moreover, raising calcium levels in the

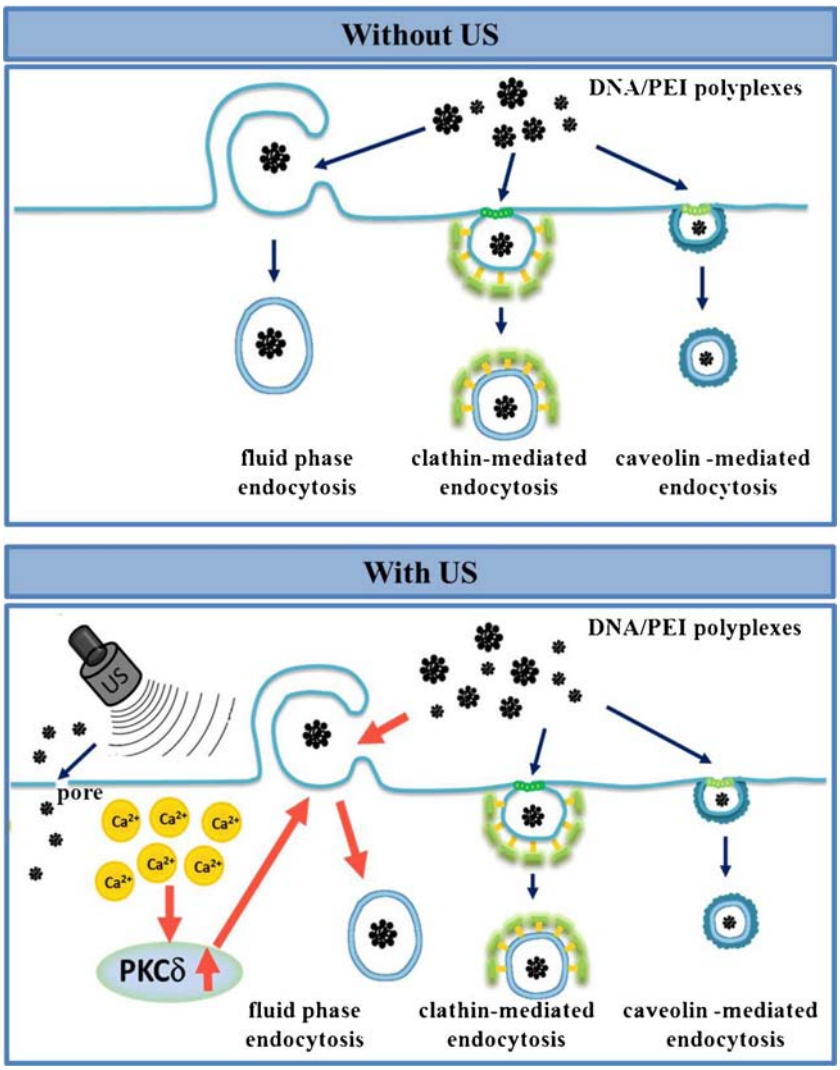

Fig. 8 Proposed detailed schematic of intracellular trafficking mechanisms for the enhancing effect of PEI-mediated gene delivery by US.

medium could dose-dependently increase the transgene expression on PEI-mediated gene transfer (Fig. 6a), while calcium channel blockers reduced the transfection levels (Figs. 6c, d and 7). Interestingly, some studies have shown that calcium could increase the endocytotic vesicle size and accelerates membrane fission (30,41,42), which may help to explain the effect of calcium on enhancing endocytosis and transgene expression. Therefore, we believe that US may enhance PEI-mediated gene transfer by activating PKC-δ activity through increasing the intracellular calcium level.

To determine whether the increase of PEI-mediated gene transfer by calcium is specific to US, lipofetamine 2000, another widely used non-viral gene transfer reagent, was tested for cmoparison. Lipofetamine 2000 is well-known to enhance gene transfer by enhancing endocytosis. Nonetheless, Fig. 6b shows that increasing the calcium levels to 10.8 mM does not significantly enhance the transgene expression when compared with the control (1.8 mM of calcium level). Thus, Lipofetamine 2000 may enhance gene transfer *via* pathways unrelated to the calcium.

In the current study, marked increase in the transgene expression could be found for the US exposure groups, and especially pronounced in the *in vivo* group (verapamil 0, Figs. 6c *vs.* 7b). US enhanced 25% of expression in the *in vitro* condition, but more than ten times in the *in vivo* condition. Similar condition has also been reported in our previous report (17). The inhibitory effect of verapamil is also significantly stronger in the *in vivo* condition. The calcium concentration in the culture medium is limited, but it is well established that skeletal muscle has abundance of calcium which can be released from the sarcoplasmic reticulum. Moreover, calcium influx is essential for the induction of PKC-δ pathway and the endocytosis of DNA/PEI polyplexes (Fig. 5). The above reasons may partly explain why US exhibits greater effect on the *in vivo* transfection.

Previous studies have reported that US-mediated gene transfer or drug delivery is related to transient pores on the cell membrane created by acoustic cavitation, a phenomenon called sonoporation (43–45). Cavitation is the oscillation of microbubbles (in the current study, SonoVue® contrast agent) in an acoustic field. It has also been reported that pores reseal a few seconds to a few minutes following the termination of US exposure (26,46,47), with pore size ranging from 200 nm to 1 μm for MCF-7 cells (45). As shown in Fig. 7, the transgene expression was not totally inhibited by verapamil. One possible explanation is that a certain number of DNA/PEI polyplexes may enter cells *via* sonoporation. Due to limited pore size, small particles such as unbound DNA may prefer to enter cells *via* these pores, while larger particles such as DNA/PEI polyplexes, internalize *via* endocytosis. Both contribute to the transgene expression but only those chose endocytotic pathways could be inhibited by verapamil. Meijering *et al.* has demonstrated that the internalization pathways after US exposure was molecular size dependent, and both transient pore formation and endocytosis play certain roles. The contribution of transient pores decreases when molecule size increases (28). In the current study, we have shown that US can facilitate the internalization of large polyplex molecules *via* endocytosis. It is well known that US can internalized smaller molecules such as naked DNA both *in vitro* and *in vivo* (11,14,16). Whether endocytosis is also involved in the internalization of naked DNA, and its percentage of contribution, need further investigation.

CONCLUSION

In conclusion, our results show that gene transfer by DNA/PEI polyplexes might mediate *via* the clathrin, caveolin, and fluid phase endocytosis (PKC-δ pathway), whereas US enhances the PEI-mediated gene transfection specifically *via* augmenting the fluid phase endocytosis, which is induced by increasing the intracellular calcium levels. The proposed detailed schematic of intracellular trafficking mechanisms for the enhancing effect of PEI-mediated gene delivery by US is summarized in Fig. 8.

ACKNOWLEDGMENTS AND DISCLOSURES

This work was supported by Grant NSC 100-2923-B-002-004-MY3 and 100-2923-B-002-003-MY3 (SonInCaRe ANR-NSC project) from the National Science Council of the Republic of China. We also thank the staff of the Biomedical Resource Core at the First Core Labs, National Taiwan University College of Medicine, for technical support.

REFERENCES

1. Duvshani-Eshet M, Benny O, Morgenstern A, Machluf M. Therapeutic ultrasound facilitates antiangiogenic gene delivery and inhibits prostate tumor growth. Mol Cancer Ther. 2007;6(8):2371–82.
2. Tamura T, Nishi T, Goto T, Takeshima H, Dev SB, Ushio Y, *et al.* Intratumoral delivery of interleukin 12 expression plasmids with in vivo electroporation is effective for colon and renal cancer. Hum Gene Ther. 2001;12(10):1265–76.
3. Felgner PL, Gadek TR, Holm M, Roman R, Chan HW, Wenz M, *et al.* Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc Natl Acad Sci U S A. 1987;84(21):7413–7.
4. Wheeler CJ, Felgner PL, Tsai YJ, Marshall J, Sukhu L, Doh SG, *et al.* A novel cationic lipid greatly enhances plasmid DNA delivery and expression in mouse lung. Proc Natl Acad Sci U S A. 1996;93(21):11454–9.
5. Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D, Demeneix B, *et al.* A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. Proc Natl Acad Sci U S A. 1995;92(16):7297–301.

6. Hayashi S, Mizuno M, Yoshida J, Nakao A. Effect of sonoporation on cationic liposome-mediated IFNbeta gene therapy for metastatic hepatic tumors of murine colon cancer. Cancer Gene Ther. 2009;16(8):638–43.
7. Feril Jr LB, Ogawa R, Kobayashi H, Kikuchi H, Kondo T. Ultrasound enhances liposome-mediated gene transfection. Ultrason Sonochem. 2005;12(6):489–93.
8. Un K, Kawakami S, Suzuki R, Maruyama K, Yamashita F, Hashida M. Development of an ultrasound-responsive and mannose-modified gene carrier for DNA vaccine therapy. Biomaterials. 2010;31(30): 7813–26.
9. Bekeredjian R, Chen SY, Frenkel PA, Grayburn PA, Shohet RV. Ultrasound-targeted microbubble destruction can repeatedly direct highly specific plasmid expression to the heart. Circulation. 2003;108(8):1022–6.
10. D'Souza AL, Tseng JR, Pauly KB, Guccione S, Rosenberg J, Gambhir SS, *et al.* A strategy for blood biomarker amplification and localization using ultrasound. Proc Natl Acad Sci U S A. 2009;106(40):17152–7.
11. Kim HJ, Greenleaf JF, Kinnick RR, Bronk JT, Bolander ME. Ultrasound-mediated transfection of mammalian cells. Hum Gene Ther. 1996;7(11):1339–46.
12. Marin A, Sun H, Husseini GA, Pitt WG, Christensen DA, Rapoport NY. Drug delivery in pluronic micelles: effect of high-frequency ultrasound on drug release from micelles and intracellular uptake. J Control Release. 2002;84(1–2):39–47.
13. Gao ZG, Fain HD, Rapoport N. Controlled and targeted tumor chemotherapy by micellar-encapsulated drug and ultrasound. J Control Release. 2005;102(1):203–22.
14. Taniyama Y, Tachibana K, Hiraoka K, Aoki M, Yamamoto S, Matsumoto K, *et al.* Development of safe and efficient novel nonviral gene transfer using ultrasound: enhancement of transfection efficiency of naked plasmid DNA in skeletal muscle. Gene Ther. 2002;9(6): 372–80.
15. Lu QL, Liang HD, Partridge T, Blomley MJ. Microbubble ultrasound improves the efficiency of gene transduction in skeletal muscle in vivo with reduced tissue damage. Gene Ther. 2003;10(5): 396–405.
16. Liao ZK, Tsai KC, Wang HT, Tseng SH, Deng WP, Chen WS, *et al.* Sonoporation-mediated anti-angiogenic gene transfer into muscle effectively regresses distant orthotopic tumors. Cancer Gene Ther. 2012;19(3):171–80.
17. Lee JL, Lo CW, Ka SM, Chen A, Chen WS. Prolonging the expression duration of ultrasound-mediated gene transfection using PEI nanoparticles. J Control Release. 2012;160(1):64–71.
18. Conner SD, Schmid SL. Regulated portals of entry into the cell. Nature. 2003;422(6927):37–44.
19. Traub LM. Clathrin couture: fashioning distinctive membrane coats at the cell surface. PLoS Biol. 2009;7(9):e1000192.
20. Richter T, Floetenmeyer M, Ferguson C, Galea J, Goh J, Lindsay MR, *et al.* High-resolution 3D quantitative analysis of caveolar ultrastructure and caveola-cytoskeleton interactions. Traffic. 2008;9(6): 893–909.
21. von Gersdorff K, Sanders NN, Vandenbroucke R, De Smedt SC, Wagner E, Ogris M. The internalization route resulting in successful gene expression depends on both cell line and polyethylenimine polyplex type. Mol Ther. 2006;14(5):745–53.
22. Rejman J, Bragonzi A, Conese M. Role of clathrin- and caveolae-mediated endocytosis in gene transfer mediated by lipo- and polyplexes. Mol Ther. 2005;12(3):468–74.
23. van der Aa MA, Huth US, Hafele SY, Schubert R, Oosting RS, Mastrobattista E, *et al.* Cellular uptake of cationic polymer-DNA complexes via caveolae plays a pivotal role in gene transfection in COS-7 cells. Pharm Res. 2007;24(8):1590–8.
24. Hufnagel H, Hakim P, Lima A, Hollfelder F. Fluid phase endocytosis contributes to transfection of DNA by PEI-25. Mol Ther. 2009;17(8): 1411–7.
25. Tachibana K, Uchida T, Ogawa K, Yamashita N, Tamura K. Induction of cell-membrane porosity by ultrasound. Lancet. 1999;353(9162):1409.
26. Mehier-Humbert S, Bettinger T, Yan F, Guy RH. Plasma membrane poration induced by ultrasound exposure: implication for drug delivery. J Control Release. 2005;104(1):213–22.
27. Juffermans LJ, Kamp O, Dijkmans PA, Visser CA, Musters RJ. Low-intensity ultrasound-exposed microbubbles provoke local hyperpolarization of the cell membrane via activation of BK(Ca) channels. Ultrasound Med Biol. 2008;34(3):502–8.
28. Meijering BD, Juffermans LJ, van Wamel A, Henning RH, Zuhorn IS, Emmer M, *et al.* Ultrasound and microbubble-targeted delivery of macromolecules is regulated by induction of endocytosis and pore formation. Circ Res. 2009;104(5):679–87.
29. Sundqvist T, Liu SM. Hydrogen peroxide stimulates endocytosis in cultured bovine aortic endothelial cells. Acta Physiol. 1993;149(2): 127–31.
30. MacDonald PE, Eliasson L, Rorsman P. Calcium increases endocytotic vesicle size and accelerates membrane fission in insulin-secreting INS-1 cells. J Cell Sci. 2005;118(Pt 24):5911–20.
31. Lo CW, Desjouy C, Chen SR, Lee JL, Inserra C, Bera JC, *et al.* Stabilizing in vitro ultrasound-mediated gene transfection by regulating cavitation. Ultrason Sonochem. 2014;21(2):833–9.
32. Tsai KC, Fang SY, Yang SJ, Shieh MJ, Lin WL, Chen WS. Time dependency of ultrasound-facilitated gene transfection. J Gene Med. 2009;11(8):729–36.
33. Tsai KC, Liao ZK, Yang SJ, Lin WL, Shieh MJ, Hwang LH, *et al.* Differences in gene expression between sonoporation in tumor and in muscle. J Gene Med. 2009;11(10):933–40.
34. Schneider M. Characteristics of SonoVuetrade mark. Echocardiography. 1999;16(7, Pt 2):743–6.
35. Vercauteren D, Vandenbroucke RE, Jones AT, Rejman J, Demeester J, De Smedt SC, *et al.* The use of inhibitors to study endocytic pathways of gene carriers: optimization and pitfalls. Mol Ther. 2010;18(3): 561–9.
36. Soltoff SP. Rottlerin: an inappropriate and ineffective inhibitor of PKCdelta. Trends Pharmacol Sci. 2007;28(9):453–8.
37. Sarkar K, Kruhlak MJ, Erlandsen SL, Shaw S. Selective inhibition by rottlerin of macropinocytosis in monocyte-derived dendritic cells. Immunology. 2005;116(4):513–24.
38. Kato K, Yamanouchi D, Esbona K, Kamiya K, Zhang F, Kent KC, *et al.* Caspase-mediated protein kinase C-delta cleavage is necessary for apoptosis of vascular smooth muscle cells. Am J Physiol Heart Circ Physiol. 2009;297(6):H2253–61.
39. Tang W, Liu Q, Wang X, Wang P, Cao B, Mi N, *et al.* Involvement of caspase 8 in apoptosis induced by ultrasound-activated hematoporphyrin in sarcoma 180 cells in vitro. J Ultrasound Med. 2008;27(4):645–56.
40. Nishizuka Y. Protein kinases.5. Protein-kinase-C and lipid signaling for sustained cellular-responses. FASEB J. 1995;9(7):484–96.
41. Artalejo CR, Henley JR, McNiven MA, Palfrey HC. Rapid endocytosis coupled to exocytosis in adrenal chromaffin cells involves Ca2+, GTP, and dynamin but not clathrin. Proc Natl Acad Sci U S A. 1995;92(18):8328–32.
42. Eliasson L, Proks P, Ammala C, Ashcroft FM, Bokvist K, Renstrom E, *et al.* Endocytosis of secretory granules in mouse pancreatic beta-cells evoked by transient elevation of cytosolic calcium. J Physiol. 1996;493(Pt 3):755–67.
43. Schlicher RK, Radhakrishna H, Tolentino TP, Apkarian RP, Zarnitsyn V, Prausnitz MR. Mechanism of intracellular delivery by acoustic cavitation. Ultrasound Med Biol. 2006;32(6):915–24.

44. Yang F, Gu N, Chen D, Xi X, Zhang D, Li Y, *et al.* Experimental study on cell self-sealing during sonoporation. J Control Release. 2008;131(3):205–10.
45. Qiu Y, Luo Y, Zhang Y, Cui W, Zhang D, Wu J, *et al.* The correlation between acoustic cavitation and sonoporation involved in ultrasound-mediated DNA transfection with polyethylenimine (PEI) in vitro. J Control Release. 2010;145(1):40–8.
46. Zhou Y, Shi JY, Cui JM, Deng CX. Effects of extracellular calcium on cell membrane resealing in sonoporation. J Control Release. 2008;126(1):34–43.
47. van Wamel A, Kooiman K, Harteveld M, Emmer M, ten Cate FJ, Versluis M, *et al.* Vibrating microbubbles poking individual cells: drug transfer into cells via sonoporation. J Control Release. 2006;112(2):149–55.