



NF- κ B related transgene expression in mouse tibial cranial muscle after pDNA injection followed or not by electrotransfer



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ABSTRACT

Background: When activated, NF- κ B can promote the nuclear import and transcription of DNA possessing NF- κ B consensus sequences. Here, we investigated whether NF- κ B is involved in the plasmid electrotransfer process.

Methods: Mouse tibial cranial muscles were transfected with plasmids encoding luciferase bearing or not NF- κ B consensus sequences. Luciferase transgene expression was evaluated noninvasively by luminescence imaging and the number of pDNA copies in the same muscles by qPCR. RT-PCR of heat shock protein Hsp70 mRNA evidenced cell stress. Western blots of phosphorylated I κ B α were studied as a marker of NF- κ B activation.

Results: Intra-muscular injection of a plasmid bearing a weak TATA-like promoter results in a very low muscle transfection level. Electrotransfer significantly increased both the number of pDNA copy and the transgene expression of this plasmid per DNA copy. Insertion of NF- κ B consensus sequences into pDNA significantly increased the level of gene expression both with and without electrotransfer. Electrotransfer-induced cellular stress was evidenced by increased Hsp70 mRNA. Phosphorylated I κ B α was slightly increased by simple pDNA injection and a little more by electrotransfer. We also observed a basal level of phosphorylated I κ B α and thus of free NF- κ B in the absence of any stimulation.

General significance: pDNA electrotransfer can increase transgene expression independently of NF- κ B. The insertion of NF- κ B consensus sequences into pDNA bearing a weak TATA-like promoter leads to enhanced transgene expression in muscle with or without gene electrotransfer. Finally, our results suggest that the basal amount of free NF- κ B in muscle might be sufficient to enhance the activity of pDNA bearing NF- κ B consensus sequences.

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1. Introduction

DNA transfer through the cell membrane is the first step towards plasmid cell transfection and transgene expression. It can be done either by direct delivery into the cytosol or via endocytosis. Once internalized plasmid DNA must overcome endo-lysosomal entrapment and cytosolic sequestration [1]. The last steps involve DNA nuclear import, transcription, and lastly mRNA translation. Optimized DNA transfer through the cell membrane is necessary for non-viral transfection methods, but the nuclear import and transcription steps are still prone to great improvements. Among the family of transcription factors only some of them such as NF- κ B operate for increasing DNA nuclear import and transcription [2].

Various copolymers of polyethylene oxide and polypropylene oxide (pluronic) often used as excipient in pharmaceutical preparation have been reported to affect various cellular function such as mitochondrial

respiration, ATP synthesis, and activity of drug efflux transporters [3] (Batrakova, JCR 2008, 130(2): 98–106). Particularly, some of them have been shown to induce transgene expression in muscle [1–4] by acting on DNA nuclear import and transcription through NF- κ B activation [3–5]. To note, pluronic are not able to transfer DNA into the cells and do not transfect in vitro [6] apart from being associated to a chemical vector [5,7]. In vivo, intramuscular DNA delivery using pluronic is more controversial [8,9].

It also has been shown that multiple virus families (including HIV-1, HBV, HCV, EBV, influenza) use the NF- κ B pathway for the transcription of several viral proteins. Like HIV-1, these viruses contain NF- κ B binding sites in their promoter and act on the cell machinery to activate the NF- κ B pathway [10]. The NF- κ B pathway usually operates to induce the transcription of genes playing a role in inflammatory processes, immune response and cell growth [11], but it can also be activated in cell stress situations [12].

Electrotransfer is an efficient technique to get DNA internalization into the cells, leading to gene expression both in vitro and in vivo. Several studies were devoted to study the mechanism of DNA electrotransfer into cells, particularly, in vitro, regarding the effects of electric pulses on

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membrane permeabilization, endocytosis, DNA movement in the cytosol [13–17]. In vivo, on the muscle model we previously studied respective effects of electroporation and DNA electrophoresis by using a combination of high voltage low duration (HV) and low voltage long duration (LV) electric pulses [9,18,19]. However, to our knowledge the further steps on nuclear import of DNA and its transcription remain to be studied. Among different processes induced by the electric field, evidence points to a reversible inflammation [20,21]. Various transcription factors are able to induce inflammatory cytokine synthesis. Among them NF- κ B and AP1 have an essential role [12,22]. Gonçalves et al. have shown that upon stimulation of the NF- κ B pathway nuclear import of pDNA bearing NF- κ B consensus sequences was favored [5]. We therefore choose to study if electrotransfer activates the NF- κ B pathway and if yes whether it impacts pDNA nuclear import and transcription.

For this purpose we compared muscle transfection of a plasmid encoding luciferase with or without NF- κ B consensus sequences (p3NF-luc, p3NF-luc-3NF and pTAL-luc) after simple DNA injection or DNA injection followed by electrotransfer. Electrotransfer was performed using a simple series of identical electric pulses which was shown to be efficient in many of our studies (see for example [23]). Transfection was evaluated noninvasively by optical imaging of muscle luminescence after injection of the luciferase substrate [24]. In addition, we evaluated the number of plasmid DNA copies in muscle fibers by qPCR the day following the bioluminescent evaluation of muscle transfection. Finally, phosphorylated I κ B α was Western-blotted in an attempt to evidence more directly NF- κ B pathway activation in transfected muscle in the absence or presence of electrotransfer.

2. Material and methods

2.1. Plasmid

Plasmid pTAL-luc, purchased from Clontech (Takara Bio Europe SAS, Saint-Germain-en-Laye, France), encodes the firefly luciferase under the control of weak TATA-like promoter region (P_{TAL}) from herpes simplex virus thymidine kinase. As described by Gonçalves et al. [5], the p3NF-luc plasmid was constructed by inserting three repeats of the kB (5'-GGGACTTTC-3') site in pTAL-luc upstream of the promoter region. In the p3NF-luc-3NF plasmid, the same 3 repeats were added downstream of the luciferase gene.

2.2. Animals

In vivo studies were performed on 6–8-week-old female BalbC/J mice and nude Swiss mice (Janvier, Le Genest-Saint-Isle, France). Before all procedures (treatment and imaging), animals were anesthetized by intraperitoneal injection of ketamine 100 mg/kg and xylazine 10 mg/kg (Bayer Pharma, Puteaux, France). The studies followed the recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimentation and the local Ethics Committee on Animal Care and Experimentation.

2.3. General experimental procedure and electric pulse delivery in vivo

For each experiment, the legs of the anesthetized mice were shaved using a depilatory cream. Then an insulin syringe (MYINJECTOR 29Gx1/2, Terumo, Leuven, Belgium) was used to longitudinally inject tibial cranial muscle with 30 μ l of a solution (NaCl 0.9%) containing pDNA coding the firefly luciferase (30 μ g or 50 μ g according to the experiment). The speed of injection was between 6 and 10 μ l/s which is in the medium range of the study of André et al. [25]. As we used the same speed of injection for the different group of mice we considered that it is possible to make direct comparisons. When required, electric pulses were delivered 20 s after plasmid DNA injection through two stainless steel plate electrodes (10 $\times$ 20 mm) placed 4 mm apart at each side of the mouse leg. Electrical contact with the shaved leg skin was ensured by means

of a conductive gel (Aquasonic 100, Parker Laboratories, Fairfield, NJ). Electric pulses were generated by a Cliniporator electropulsator (IGEA, Carpi, Italy). Our electrotransfer (ET) procedure (8 pulses of 20 msec duration at 190 V/cm at 2 Hz) has been used as an efficient condition in many of our previous studies (see for example [23]) and can be performed with classical electrotransfer device.

2.4. In vivo optical imaging of luciferase activity

Luciferin potassium salt (SYNCHÉM, Felsberg/Altenburg, Germany) diluted in PBS was injected locally into the tibial cranial muscle of the anesthetized mice at a dose of 100 μ g/40 μ l, which is way above the relative amount of luciferase [24]. Optical imaging used a cooled intensified charge-coupled device (CCD) camera (Biospace, Photon Imager, Paris, France) placed in a black box. Luminescence level was measured in regions of interest (ROIs) corresponding to the tibial cranial muscle as described previously [24]. We used the same ROI from one experiment to another. We opted to take the mean values in cpm of all the measurements at 10 min after the start of acquisition [24]. Optical imaging of luminescence was performed at different time points between day 2 and day 29 post-DNA injection. In a previous work we verified that after one hour delay the subsequent injection of the same amount of luciferin induced similar luminescence production [24]. Consequently, we assume that the measurement of luciferase activity at a given time was not altered by previous measurements. When necessary legs of anesthetized mice were shaved again before optical imaging.

2.5. In vitro measurement of muscle luciferase activity

For experiments with nude mice, muscle luciferase activity was measured in vitro on muscle extract according to a procedure previously described [6]. Briefly, mice were killed 7 days post-administration and muscles were dissected and snap-frozen in liquid nitrogen. For the measurement of luciferase levels in muscle, the following protocol was used: lysis buffer (500 μ l) containing a cocktail of protease inhibitors (Sigma) was added to the collected organs. Each organ was then homogenized for approximately 30 s with an Ultra-turrax (Ika, Staufen, Germany), and the homogenate was centrifuged for 10 min at 8000 g at 4 °C. A 5- μ l aliquot of the supernatant was used for the luciferase assay. Protein content was measured by using the Bradford protein assay. Luciferase background was subtracted from each value and the transfection efficiency is expressed as total light units/10 s/mg protein.

2.6. Muscle sample collection for in vitro measurements

Mice were euthanized by asphyxia in a box containing dry ice (solid carbon dioxide), and the tibial cranial muscle was then withdrawn and immersed in liquid nitrogen to freeze. Samples were then stored at –80 °C until analysis.

2.7. DNA extraction from muscles

For DNA extraction, the muscles used to evaluate transfection were collected one day after optical imaging of the mice, i.e. 3 days post-DNA injection. Each muscle was homogenized with 1 ml of DNAzol then centrifuged 10 min at 10,000 g, at 4 °C. The viscous supernatant was transferred into a new tube. We then precipitated pDNA by adding 0.5 ml ethanol 100%. The pellet was washed with ethanol 75% then solubilized in NaOH 8 mM.

2.8. qPCR

The number of copies of plasmid DNA pTAL-luc or p3NF-luc-3NF was quantified by qPCR on muscle extract. We used the primer sense (5'-CCAGGGATTTCAGTCGATGT-3') and antisense (5'-AGAATCTCACGCAGGCAGTT-3') interacting with luciferase coding part of both plasmid

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