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NF-kB 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-κB can promote the nuclear import and transcription of DNA possessing NF-κB consensus sequences. Here, we investigated whether NF-κB is involved in the plasmid electrotransfer process.
Methods: Mouse tibial cranial muscles were transfected with plasmids encoding luciferase bearing or not NF-κB 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 IkBα were studied as a marker of NF-κB 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-κB 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 IκBα was slightly increased by simple pDNA injection and a little more by electrotransfer. We also observed a basal level of phosphorylated IκBα and thus of free NF-κB in the absence of any stimulation.
General significance: pDNA electrotransfer can increase transgene expression independently of NF-κB. The insertion of NF-κB 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-κB in muscle might be sufficient to enhance the activity of pDNA bearing NF-κB consensus sequences.

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-κB operate for increasing DNA nuclear import and transcription [2]

Various copolymers of polyethylene oxide and polypropylene oxide (pluronics) 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-κB 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-κB pathway for the transcription of several viral proteins. Like HIV-1, these viruses contain NF-kB binding sites in their promoter and act on the cell machinery to activate the NF-κB pathway [10]. The NF-κB 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 electropermeabilization 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-κB and AP1 have an essential role [12,22]. Gonçalves et al. have shown that upon stimulation of the NF-κB pathway nuclear import of pDNA bearing NF-κB consensus sequences was favored [5]. We therefore choose to study if electrotransfer activates the NF-κB 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-κB 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 IκBα was Western-blotted in an attempt to evidence more directly NF-κB 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′-GGGACTTTCC-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 μl of a solution (NaCl 0.9%) containing pDNA coding the firefly luciferase (30 μg or 50 μg according to the experiment). The speed of injection was between 6 and 10 μl/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 × 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 (SYNCHEM, Felsberg/Altenburg, Germany) diluted in PBS was injected locally into the tibial cranial muscle of the anesthetized mice at a dose of 100 μg/40 μl, 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 μl) 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-μl 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′-AGAATCTCACGC AGGCAGTT-3′) interacting with luciferase coding part of both plasmid

DNA. For each sample, DNA was diluted (H_2O) in order to reach 50 ng of DNA in 3 μl then mixed with Taq polymerase (2.5 μl), dNTP (2.5 μl), sybr green (2.5 μl), buffer 10x (2.5 μl), sense and antisense sequences ([10 μM], 0.5 μl), and H_2O in a final volume of 25 μl. The standard curve to determine the amount of plasmid DNA copies in each sample from fluorescence was obtained by successive dilutions of pTAL-luc DNA (10^8 copies). The number of copies n was calculated using the equation:

$$n = \left(\text{amount in ng} * 6.022\ 10^{23}\right) / \left(\text{length (in bp)} * 10^9 * 650\right).$$

2.9. RNA extraction from muscle

Each muscle was homogenized with RNABLe® according to the manufacturer's instructions. The RNABLe® suspension was then mixed with chloroform. After centrifugation, the aqueous phase contains exclusively RNA while the phenol phase and interphase contain proteins and DNA. RNA in the aqueous phase was precipitated by addition of isopropanol. The pellet was washed with ethanol 75% (prepared with ultrapure water) then solubilized in ultrapure water. To verify that extracted RNA was not degraded, we ran electrophoresis of extracted RNA for each muscle. RNA concentration of each sample was then evaluated from fluorescence after adding Ribogreen and comparing against a calibration curve.

2.10. HSP70 mRNA level measured by quantitative real-time RT-PCR

One μg of RNA was reverse-transcribed using reverse transcriptase SSIIRT. With the reverse transcriptase and RNA, the RT-PCR mix for one sample contains some dNTPs, oligo DTT, random primers and RNaseOUT. The different RT-PCR steps were:

1- reagent incubation at 25 °C during 10 min
2- reverse transcription at 42 °C during 30 min
3- inactivation of the reverse transcriptase enzyme at 99 °C during 5 min
4- incubation at 15 °C

The number of cDNA copies matching to mRNA of HsP70 and GAPDH as reference was quantified by qPCR. For HsP70, primers, sense (5′-CTGGCTCTCCCGGTGTGGTCT-3′) and antisense (5′-ACAGTA ATCGGTGCCCAAGCAGCT-3′) were used. For GADPH primers, sense (5′-AAGATGGTGATGGGCTTCCCG-3′) and antisense (5′-TGGCAAAGTG GAGATTGTTGCC-3′) were used. The sense and antisense primers of HsP70 or GADPH were added to the PCR SYBR® Green mix. Each sample was amplified in triplicate.

2.11. Western blotting to monitor phosphorylated IκBα

After thawing, each muscle sample was lysed by mechanical crushing in 579 μl of RIPA buffer (Sigma, P0278, USA) added with 6 μl of a protease inhibitor cocktail (Sigma, P8340 USA, diluted 1:100) and 15 μl of phosphatase inhibitor (Sodium Orthovanadate, New England Biolab P0758, diluted 1:40). Samples were kept in ice for 30 min then sonicated for 15 s at a power of 3 W. Proteins were then denatured at 95 °C for 3 min. After centrifugation (12,000 *g*, 10 min, 4 °C), the supernatant was recovered and its protein concentration was evaluated using the Thermo Scientific Pierce, 23225, BCA protein assay kit. The calibration curve was established with bovine serum albumin. Knowing the protein concentration of each sample, the volume needed to obtain 30 μg protein was determined and completed with H_2O, denaturation buffer (Invitrogen, NuPAGE®Sample Reducing Agent 10X, NP0004) and charge buffer (Invitrogen, NuPAGE®LDS Sample Buffer 4X, NP0007). A repeat protein denaturation step was performed at 90 °C for 10 min.

Protein electrophoresis was performed with a Novex®NuPAGE gel system, 4–12% Bis Tris, placed in a vertical container (XCell SureLock, Invitrogen) immersed in migration buffer provided by Invitrogen (50 mM morpholino propane sulfonic, 0.1% SDS, 1 mM EDTA, 50 mM Tris base, pH 7.7). A molecular weight marker was placed in one well (SeeBlue®Plus2 Pre-Strained Standard, Invitrogen). Electrophoresis was performed for 2 h at 120 V. The gel system was then electrotransferred to a Hybond ECL membrane (Amersham Pharmacia Biotech, Piscataway, NJ) for 50 min at 25 V with a 0.45 μm pore size. Free sites of the membrane matrix were saturated with a blocking buffer (Odyssey, LI-COR, NE) completed with Tween-20 0.1%, and incubated with primary antibody diluted 1:1000 overnight at 4 °C (5 ml buffer per membrane), i.e. rabbit polyclonal antibody against phosphorylated IκBα (Abcam®, ab5682). The membranes were then washed (Tris buffered saline + Tween-20 0.1%) once for 20 min then twice for 5 min. Next, membranes were incubated with secondary antibody diluted 1:15000 in blocking buffer completed with Tween-20 at 0.1%, i.e. donkey anti-rabbit IgG coupled with IRDye 800 CW (λex 778 nm, λem 795 nm, LI-COR 926-32213).

Fluorescence imaging of the membranes was performed with an ODYSSEY®CLx scanner (LI-COR, NE). Fluorescence intensity was measured in ROIs defined on bands using ImageJ (http://rsbweb.nih.gov/ij/).

The membranes were kept at 4 °C and then stripped with 100 mM beta-mercaptoethanol, 2% SDS and 62.5 mM Tris–HCl, for 1 h at 60 °C. Saturation was achieved as described above. Membranes were incubated with rabbit anti-actin primary antibody diluted 1:1000 (Sigma-Aldrich®, A2066) in blocking buffer with Tween 20 0.1%. After repeated washes, the membranes were incubated with the secondary antibody. Scan membranes were also obtained by ODYSSEY®CLx.

2.11.1. Peptide competition experiment

The antibody raised against phosphorylated IκBα is one of the Phosphorylation Site Specific Antibodies (PSSAs). The specificity of a PSSA in each experimental system can be confirmed through peptide competition. In this experiment, a western blot was performed as previously up until the saturation part. Anti-phosphorylated IκBα antibody was used at a dilution of 1:1000 and pre-incubated with different concentrations of the peptide containing the sequence of the phosphopeptide immunogen used to raise the PSSA (Abcam®, ab10790). The phosphorylated IκBα peptide was used at 333 nM, 200 nM and 67 nM, in order to test the different molar excess peptides compared to antibody.

2.12. Statistical analysis of results

Given the non-Gaussian distribution of the results, we used non-parametric tests, i.e. Kruskal–Wallis nonparametric variance analysis of the measured parameters and a pairwise Wilcoxon test for comparisons between treatments. The statistics software used was R (R Project for Statistical Computing).

3. Results

3.1. Total transgene expression in transfected muscle

We first compared the efficacy of transfection in the mouse tibial cranial muscle injected with 30 μg/30 μl of three different plasmid constructs bearing or not NF-κB consensus sequences (p3NF-luc, p3NF-luc-3NF and pTAL-luc) (Fig. 1).

Relative to the pTAL-luc plasmid, the p3NF-luc and p3NF-luc-3NF plasmids displayed significantly increased transgene expression at days 2, 3 and 6 after plasmid DNA injection. However, no significant differences were observed between p3NF-luc and p3NF-luc-3NF, contrary

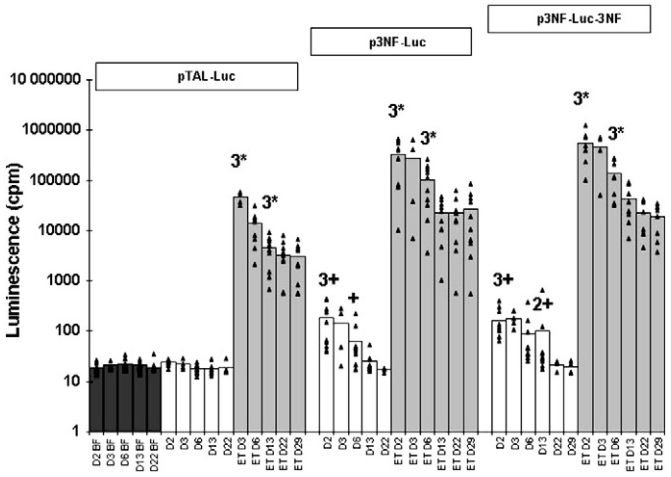

Fig. 1. Transgene expression after pTAL-luc, p3NF-luc or p3NF-luc-3NF plasmid i.m. administration with or without electrotransfer. Mean values at different times after plasmid DNA i.m. administration are given in columns and individual values for each muscle are plotted as black triangles. Dark gray columns show background values. Under each plasmid DNA (pTAL-luc, p3NF-luc, p3NF-luc-3NF), empty columns are mean transfection (luminescence) values after simple plasmid DNA i.m. administration, and grayed columns are mean transfection values after DNA injection followed by electrotransfer. Statistics: The relative transfection levels for the different conditions were very similar at each time point after plasmid DNA i.m. administration, so statistics are only given for days 2 and 13. For each plasmid DNA comparison between simple i.m. administration and electrotransfer: 3* at $p < 0.001$. Comparison with pTAL: 3+ $p \leq 0.001$, 2+ $p \leq 0.01$, + $p \leq 0.5$.

to what had been observed in vitro on HeLa cells transfected with FUGENE6 in the presence of TNFα or in mouse liver after hydrodynamic injection [5]. Electrotransfer led to highly amplified expression for all the plasmid DNAs (pTAL-luc, p3NF-luc, p3NF-luc-3NF). Similar to what was observed with simple plasmid DNA injection, a higher level of luciferase activity was observed with the two plasmids bearing NF-κB consensus sequences p3NF-luc and p3NF-luc-3NF than with pTAL-luc plasmid. As for simple DNA injection, the presence of 3 or 6 NF-κB consensus sequences did not lead to significant differences in gene expression. In terms of global pattern, whatever the treatment, expression levels decreased between 3 and 29 days after plasmid injection.

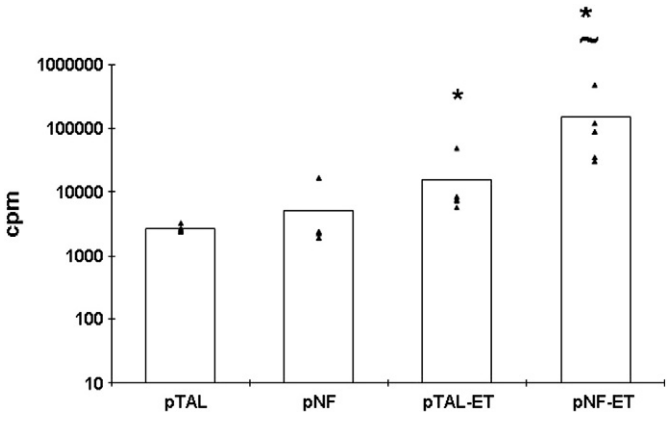

Fig. 2. Transgene expression after pTAL-Luc or pNF-κB-Luc plasmid i.m. administration with or without electrotransfer in nude mice. Mean values at different times after plasmid DNA i.m. administration are given in columns and individual values for each muscle are plotted as black triangles. pTAL: pTAL-luc plasmid, pNF: pNF-κB-luc plasmid, pTAL-ET: electrotransfer of pTAL-luc plasmid, pNF-ET: electrotransfer of pNF-κB-luc plasmid. Statistics: Significance of the difference relative to values obtained with pTAL: * at $p \leq 0.05$; relative to values obtained with pTAL-ET ~ at $p = 0.09$.

We also performed transfection experiments with immunodeficient swiss nude mice (Fig. 2). The amount of plasmid DNA injected was of 50 μg per muscle and luciferase activity was measured on muscle homogenate in vitro 7 days after plasmid injection. Luciferase activity was similar for mice injected with pTAL-Luc or pNF-κB-Luc. Using electrotransfer allowed amplification of transfection with both plasmids which was higher when using pNF-κB-Luc. This last result is comparable to the results obtained in the previous experiment with BalbC/J mice.

3.2. Number of plasmid copies and transgene expression per plasmid copies in muscle fibers

Mouse tibial cranial muscles were injected with 50 μg/30 μl of the plasmid pTAL-luc devoid of NF-κB consensus sequences, and with the plasmid p3NF-luc-3NF containing 6 NF-κB consensus sequences, followed or not by electrotransfer. Luciferase expression was evaluated noninvasively by optical imaging of luminescence at day 2. At day 3, mice were euthanized and their tibial cranial muscles collected for qPCR evaluation of the number of plasmid DNA copies in muscle fibers. We confirmed the results of Fig. 1 that electrotransfer amplified the expression of both plasmids, and that p3NF-luc-3NF led to higher expression than pTAL-luc (Fig. 3). The number of plasmid DNA copies was also increased by electrotransfer for pTAL-luc and p3NF-luc-3NF, although the difference was not statistically significant for p3NF-luc-3NF. Lastly, the level of luciferase expression per plasmid DNA copy in each muscle was clearly increased with electrotransfer or using p3NF-luc-3NF relative to pTAL-luc. The combination of p3NF-luc-3NF plus electrotransfer yielded both the highest global level of luciferase expression and the highest level of expression per DNA copy. See Table 1 for complete statistics in supplementary data.

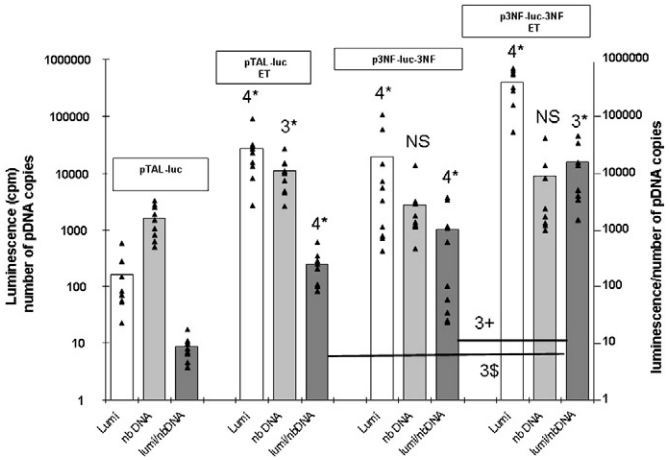

Fig. 3. Transgene expression and plasmid copy number evaluation in tibial cranial muscle after pTAL-luc or p3NF-luc-3NF plasmid i.m. administration with or without electrotransfer. Mean values at different times after pDNA i.m. administration are given in columns and individual values for each muscle are plotted as black triangles. Under each plasmid DNA (pTal-luc, p3NF-luc-3NF), empty columns represent the mean luminescence values as an index of luciferase expression, light gray columns represent the mean number of plasmid DNA copies per muscle as evaluated by qPCR, and dark gray columns represent mean luciferase expression over DNA copies, i.e. the ratio of luminescence to number of pDNA copies. Values on the y-axis chart luminescence in cpm, number of pDNA copies per muscle, and ratio of luminescence to number of pDNA copies. Statistics: Significance of the difference relative to values obtained with pTal-luc plasmid: 4* at $p \leq 0.0001$, 3* at $p \leq 0.001$. For the ratio of luminescence to number of pDNA copies, significance of the difference between p3NF-luc-3NF ET and p3NF-luc-3NF 3+ at $p \leq 0.001$; between p3NF-luc-3NF ET and pTal-luc 3$ at $p \leq 0.001$.

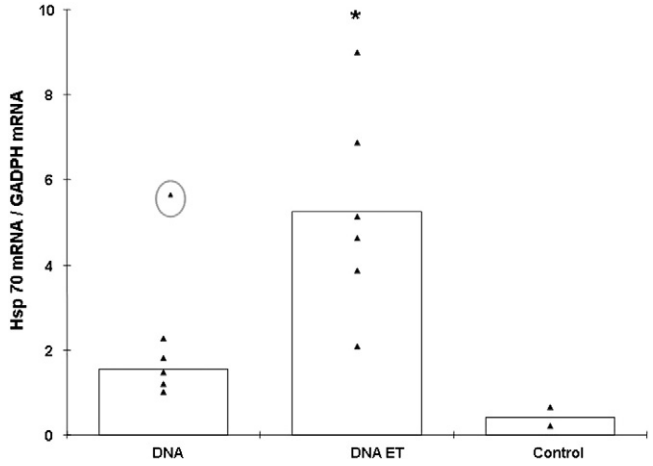

Fig. 4. Effect of p3NF-luc-3NF administration into tibial cranial muscle followed or not by electrotransfer on Hsp70 mRNA levels. Mean values at 24 h after p3NF-luc-3NF i.m. administration are given in columns and individual values for each muscle are plotted as black triangles. Values for Hsp70 mRNA were normalized to those for GADPH mRNA. The highest value measured after simple pDNA injection, surrounded by a circle, was considered aberrant and eliminated. Statistics: Significance of the difference between simple pDNA injection and pDNA injection followed by electrotransfer: * at $p \leq 0.05$.

3.3. Cell stress evaluation

The induction of heat-shock proteins, especially Hsp70, is a cellular mechanism triggered in response to stress. A wide spectrum of physical, chemical and physiological stimuli has been proven to induce Hsp70 [26–28]. Furthermore, the Hsp70 response has been shown to depend on intensity of stimuli: for instance, exercise induced a Hsp70 response in skeletal muscle [29]. Hojman et al. used a semi-quantitative approach to show that Hsp70 mRNA was increased in mouse muscle 24 and 48 h after electrotransfer [27]. Here, mRNA was evaluated by RT-qPCR. Fig. 4 shows that 24 h after p3NF-luc-3NF injection into tibial cranial muscle, the HsP70 mRNA level, as normalized to GADPH mRNA, was higher after electrotransfer compared to simple DNA injection and to control muscle. Less mRNA was detected at earlier time points (data not shown). However, simple pDNA injection increased HsP70 mRNA compared to untreated muscle, although to a lesser extent.

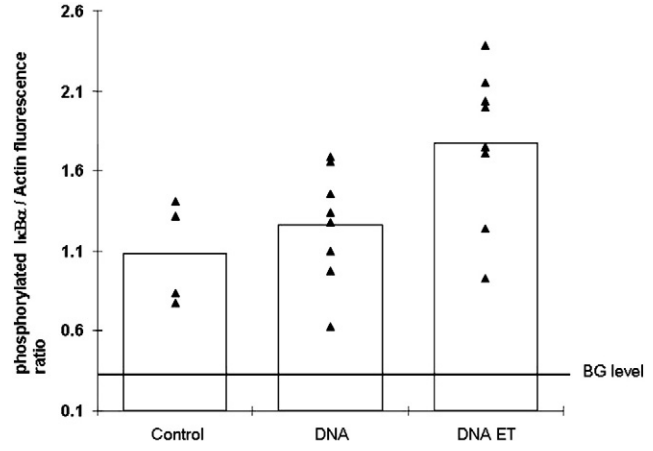

Fig. 5. Effect of p3NF-luc3NF injection into tibial cranial muscle followed or not by electrotransfer on phosphorylated IkBα. Mean values of fluorescence revealing phosphorylated IkBα are given in columns and individual values for each muscle are plotted as black triangles. Values were normalized to those for actin. By competitive inhibition with a phosphorylated IkBα peptide, the ratio of fluorescence relative to that for actin decreased to 0.315 ± 0.085 SD. This background value is indicated by a line in the figure. Statistics did not show significant differences between treatments.

3.4. Activation of the NF-κB pathway

NF-κB is sequestered in the cytosol of unstimulated cells via non-covalent interactions with I-κB. When activation occurs, the IKK (I KappaB Kinase) complex phosphorylates IκB which is released into the cytoplasm with NF-κB. IκB is subsequently degraded, enabling free NF-κB to interact with consensus DNA sequences [30,31].

We used western blotting to evaluate the fluorescence associated to phosphorylated IκBα and normalized this fluorescence to that of actin. Muscle samples were collected immediately or 2 h after p3NF-luc-3NF injection followed or not by electrotransfer. We assumed that the nature of the plasmid, particularly the presence or lack of NF-κB consensus sequences, would not interfere with the level of phosphorylated IκBα. Since levels of fluorescence associated with phosphorylated IκBα at the two collection times were very similar, we mixed them. Based on the data obtained (Fig. 5), it emerged that electrotransfer tends to increase phosphorylated IκBα but not statistically significant ($p = 0.11$) (Fig. 6). With the two muscle samples giving the highest ratio of fluorescence phosphorylated IκBα/actin, we performed competitive inhibition with the IκBα phosphorylated peptide. The fluorescence ratio measured was near background (ratio vs actin = 0.32 ± 0.08 SD) and significantly below values obtained in the absence of treatment and after simple DNA injection or DNA injection followed by electrotransfer. This indicates the presence of phosphorylated IκBα even in non-treated muscles.

4. Discussion

The main aim of this study was to determine whether the transcription factor NFκB plays a role in transgene expression in muscle after transfection by electrotransfer.

4.1. Transgene expression

In immunocompetent balb CJ mice, inserting NF-κB consensus sequences in plasmid enhances transgene expression after either DNA injection alone or followed by electrotransfer. This raised the following questions: does plasmid injection induces enough stress to activate the NF-κB pathway or does basal NF-κB activity in muscle fibers is sufficient to activate transcription.

In athymic nude Swiss mice, electrotransfer increased the expression of Clontech (Palo Alto, CA) pNF-κB-Luc. We found similar results on immunocompetent mice. The athymic nude mouse has a defective immune system lacking many immune and inflammation-related components. The fact that NF-κB is involved in inflammatory events points to the conclusion that electrotransfer enhances plasmid transfection independently of inflammation signaling pathways. This is in sharp contrast with the results obtained by Yang et al. who found that no transfection occurred in nude mice using the Pluronic P85 [4].

4.2. HsP70

RT-qPCR showed more HsP70 mRNA after electrotransfer than after simple pDNA administration, which is consistent with results of Hojman et al. using comparable electrotransfer conditions here [27]. However, it seems that simple pDNA injection also increased HsP70 mRNA in non-electrotransferred muscle. Thus, simple pDNA administration likely induces a stress that could activate the NF-κB pathway.

4.3. Nuclear import

The number of pDNA copies in muscle tissue was determined 3 days post-i.m. administration. It can be considered that this time period is long enough to have resulted in total degradation of extracellular pDNA [32,33]. In the muscle the number of pDNA copies was reduced by 4 logs 48 h after injection and probably more at 3 days [34]. Half-life of pDNA in vitro was found to be 50 min in the cytosol of COS-1

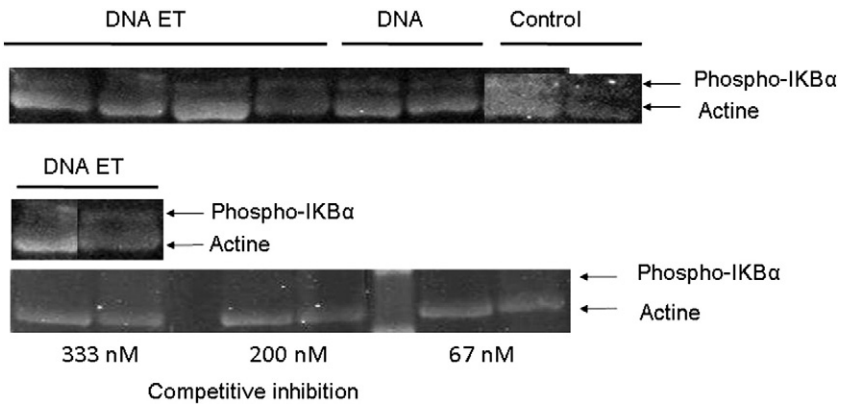

Fig. 6. Western blots of phosphorylated IkBα and actin in muscle extracts. For all lane fluorescence of antibody against phosphorylated IkBα and actin in muscles extract. Top lane 1 DNA ET: muscle injected with p3NF-luc-3NF followed by electrotransfer; DNA: muscles injected with p3NF-luc-3NF; Control: non-injected muscles. Lane 2 DNA ET: western blot of the two muscle extract that will be used to test competitive inhibition with phosphorylated IκBα peptide. Lane 3 Competitive inhibition on extracts of muscle injected with p3NF-luc-3NF followed by electrotransfer. The phosphorylated IκBα peptide was used at concentrations of 333 nM, 200 nM and 67 nM.

cells and 90 min in Hela cells [35]. In addition, the study of M Dupuis et al. showed that 24 h after electrotransfer of fluorescent pDNA into muscle, fluorescence of pDNA was only detectable in fiber nucleus [36]. Consequently, we hypothesized that the number of pDNA copies in muscle tissue as evaluated by qPCR was in majority nuclear. The number of pDNA copies did not significantly differ between pTAL-luc and p3NF-luc3NF plasmids after simple i.m. administration, suggesting that plasmid nuclear import is independent of the presence of NF-κB consensus sequences. Such a behavior was observed when NF-κB consensus sequences were associated to a strong promoter [37] but is in contradiction with earlier results reported by Gonçalves et al. [5] who used the same plasmid bearing a weak TATA like promoter. This discrepancy might originate from the fact that in that study, pDNA was delivered via chemical vectors, such as Fugene6 or polyethyleneimine and after stimulation of the NF-κB pathway by TNFα. In vivo, transfection was achieved via the hydrodynamic injection of plasmid in liver which favor NF-κB pathway activation [38].

Given that the pTAL-luc plasmid is devoid of a DNA targeting sequence (DTS) (http://www.addgene.org/vector-database/4345/), our hypothesis is that the plasmid load delivered to the myofiber cytoplasm was sufficient to obtain passive diffusion through nuclear pores. For single muscular fibers of intact mice, Utvik et al. [39] reported a threshold level of 10^6 pDNA copies to bring at least 1 copy into the nucleus. Here, we did not measure luciferase expression after injecting 30 μg of pTAL-luc plasmid into the muscle whereas luciferase expression was measurable after injecting 50 μg of the same plasmid.

This could be explained by the fact that with 50 μg, the amount of plasmid needed for myofibers to passively diffuse into the nucleus had been reached (see Figs. 1 and 3). Taken together, our results suggest that electrotransfer increased the number of plasmids in the nucleus of muscle cells in an NF-κB independent manner. This is probably due to the fact that electrotransfer allows a better delivery of DNA into the myofibers, and in turn increases the probability of passive nuclear delivery of pDNA.

4.4. Transcription

A methodological originality of our present study was the possibility to evaluate pDNA expression and number of pDNA copies in paired experiments on the same muscles, which to our knowledge has never been done before. Our hypothesis is that at the time of measurement pDNA was nuclear (see previous paragraph). Plasmidic luciferase expression was evaluated noninvasively by optical imaging of luminescence after luciferin injection. This was done two days after plasmid DNA i.m. administration, i.e. 1 day before qPCR on muscle extracts. In these experiments, expression per DNA copy was significantly higher with p3N-luc-3NF than pTAL-luc, which is consistent with an increased transcription.

After electrotransfer of pTAL-luc, expression per pDNA copy was enhanced. Thus plasmid transcription or mRNA translation appeared to be stimulated by the delivery of electric pulses independently of NF-κB. This was confirmed with the p3NF-luc-3NF plasmid. The mechanism underpinning this stimulation remains to be clarified.

4.5. Western blot of phosphorylated IκBα

To determine more directly whether the NF-κB pathway was activated, we evaluated the level of phosphorylated IκBα in muscle tissue by western blotting. Relatively to non-treated muscle, phosphorylated IκBα was slightly increased by simple pDNA i.m. injection and further increased by electrotransfer. However, none of these variations were statistically significant. We verified that our antibodies specifically revealed the presence of phosphorylated IκBα in muscle tissue by performing competitive inhibition with a phosphorylated IκBα peptide. The high significant decrease in fluorescence observed indicates the existence of a basal level of phosphorylated IκBα and thus of free NF-κB in the control group, as expected based on previous research [40,41].

In conclusion, we evidenced the usefulness of NF-κB consensus sequences for improving transgene expression in muscle with or without electrotransfer. In this tissue, we did not detect a significant increase of phosphorylated IκBα and consequently of NF-κB activation after DNA injection or DNA injection then electrotransfer relative to control group. However, phosphorylated IκBα level was clearly above the background in all groups. Consequently enough free NF-κB might be available to interact with NF-κB consensus sequences of pDNA. Electrotransfer can also increase the efficiency of pDNA expression independently of NF-κB.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2014.06.013.

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