In vitro and in vivo effects of glucocorticoids on gene transfer to skeletal muscle

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Abstract As a pharmacological approach to potentially improve gene transfer efficiency into skeletal muscle cells, glucocorticoids were shown here to allow efficient transfection of cultured and mouse human myoblasts, human pulmonary A549 cells, but not dog myoblasts, independently of the transfection protocol, the reporter gene and the transcription promoter employed. Transduction with adenovirus was also increased by dexamethasone. Pretreatment of cells 48 h prior to transfection was most effective and was shown to be concentration-dependent. This effect is mediated by binding to the glucocorticoid receptor, but not by glucocorticoid responsive elements present in the vectors. The acute dexamethasone effect could be due to increased plasmid entry into the cells as suggested by Southern blot, whereas the sustained increase of luciferase activity in dexamethasone-treated cultures may be related to intracellular mechanisms following cell entry. In mice in vivo, a similar increase of luciferase activity upon glucocorticoid treatment was found.

Key words: Glucocorticoid; Skeletal muscle; Myoblast; Gene transfer

1. Introduction

The level of gene transfection efficiency is currently too low to envisage gene therapy for disorders affecting muscles, e.g. Duchenne muscular dystrophy (DMD), for which it is necessary to introduce the wild type dystrophin gene into a minimum of 20% of the myofibers [1]. To obtain functional correction of the muscles, different methods of gene transfer are being developed [2]. This includes naked DNA, synthetic vectors, recombinant viruses [3–6] or engineered precursor cell grafts [1,7,8]. Besides vector design, it is also necessary to increase transfection or transduction efficiencies [9] to allow clinical development.

Human skeletal muscle myoblasts are extremely difficult to transfect in vitro by either calcium phosphate precipitation or lipid-complexed plasmids. To our knowledge no report of efficient in vitro transfection of human myoblasts has been published. In contrast, human myoblast transduction with viral vectors is more effective [10–12]. Cultured human myoblasts may serve as a valuable model to study pharmacological approaches for improving gene transfer efficiency.

Glucocorticoid hormones have different physiological functions, including effects on metabolism and the immune system. They are widely used clinically as immunosuppressive anti-inflammatory agents. The biological effects of glucocorticoids are generally thought to be mediated by an intracellular (cytoplasmic) receptor protein, the glucocorticoid receptor (GR). Upon steroid binding, the GR dissociates from its protein complex, enters the nucleus, dimerizes and binds to specific DNA sequences called glucocorticoid responsive elements (GRE), which participate positively or negatively in gene regulation [13,14]. Glucocorticoids mediate their effects by influencing gene expression and protein synthesis at essentially all known levels of regulation. Due to their lipophilicity, glucocorticoids may also modify membrane fluidity [15]. The various known effects of glucocorticoids (gene regulation, alteration of membrane fluidity, reduction of inflammation and immunity) may thus represent a successful way to improve gene transfer to cells ex vivo and in vivo. Those properties combined with the known trophic effects of glucocorticoids on skeletal muscle cells of various species (see for example [16,17]) and the clinical benefit provided by glucocorticoids in DMD [18,19] led us to investigate their potential in gene transfer into skeletal muscle cells.

2. Materials and methods

2.1. Materials

Dexamethasone, cortisol, indomethacin, nordihydroguaiaretic acid, quinacrine, and ionomycin were purchased from Sigma Chemicals (St. Louis, MO, USA); RU38486 was provided by Bruno Dumas (Roussel UCLAF, Romainville, France). α-Methylprednisolone (Solumedrol) was from Upjohn (Paris, France). Concentrations used were determined according to previously published data on biological effects on cell cultures including human myoblasts [16,17].

2.2. Cell cultures

Primary satellite cells (myoblasts). Muscle cultures were established from satellite cells of portions of muscle biopsies: (a) control patients X and Y: spinal muscle biopsies of two 15-year-old girls who had undergone orthopedic surgery, (b) spinal muscle biopsy of a 14-year-old male diagnosed with DMD, (c) mouse C57BL/10 quadriceps muscle, and (d) dystrophic Golden retriever dog extensor digitorum longus muscle. Except for mouse muscles that were subjected to enzymatic digestion, cells were harvested from explant cultures and grown in Ham's F14 medium (Life Technologies) supplemented

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Abbreviations: GR, glucocorticoid receptor; GRE, glucocorticoid responsive elements; DMD, Duchenne muscular dystrophy; i.m., intramuscular

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with 10% fetal calf serum (Hyclone, Logan, UT, USA), 10 μg/ml insulin, 10 ng/ml epidermal growth factor (both from Sigma), 10 ng/ml basic fibroblast growth factor (PeproTech, Rocky Hill, NJ, USA), 2 mM glutamine (bioMérieux, Marcy l'Etoile, France) and 40 μg/ml gentamicin (Schering-Plough, Kenilworth, NJ, USA) [20,21]. Experiments were performed on myoblasts obtained after a maximum of 12 passages and seeded (10^4 cells/cm^2) on 0.1% gelatin-coated supports. Culture supports were either 96, 24 or 6 microwell plates (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA), depending on the experimental design. The muscle phenotype of the primary cells was determined by immunocytochemistry for desmin, dystrophin (NCL anti-dystrophin monoclonal antibodies, Novocastra) and their ability to fuse to form myotubes. Proportions of myoblasts in the cultures used for our experiments were between 70 and 90%.

A549 cells *(ATCC CCL 185, epithelial cells derived from a human pulmonary carcinoma)* were cultured in (DMEM) containing 10% fetal calf serum (Life Technologies, Cergy-Pontoise, France).

2.3. Vectors and gene transfer procedures

2.3.1. Plasmids. The plasmids used for the transfection experiments were: pTG11033, an *Escherichia coli* plasmid, based on the ColE1 origin of replication. It carries an expression cassette encoding the firefly luciferase gene [22] with the mouse HMGCR intron [23] and driven by the human cytomegalovirus IE1 promoter (from pCEP4, InVitrogen, Abingdon, UK). pTG11034 has the same backbone as pTG11033, and carries a *lacZ* gene with a nuclear localization signal instead of the luciferase gene. pTG11174 is a derivative of pTG11033 resulting from the replacement of the CMV IE1 promoter by the RSV promoter (from pREP4, InVitrogen). pTG11056 contains the same luciferase expression cassette as pTG11033 and is devoid of any GRE sequence. pTG11096 is a derivative of pTG11056 with the U3 domain from the LTR of the Friend murine leukemia virus (FB29 strain [24]) and a GRE sequence about 600 bp upstream to the polyadenylation signal of the luciferase expression cassette. pCH110N, generously provided by Prof. Pierre Chambon (IGBMC, Strasbourg, France), derives from pCH110 [25]; it carries the *lacZ* gene with a nuclear localization signal and under the control of the early promoter of SV40. Plasmids were amplified in *E. coli* strain MC1061 and purified by double CsCl gradient centrifugation after alkaline lysis according to standard techniques [26]. Purified plasmid DNA was concentrated to 1 mg/ml in 10 mM Tris, 1 mM EDTA, pH 7.5.

Calcium phosphate transfections were performed according to the standard protocol [27] on either adherent (0.5, 2 or 1 μg plasmid DNA/well of 96 or 24 well plates or 8 well Lab-Tek tissue culture chambers (Nunc, Naperville, IL, USA) or suspended cells (obtained by trypsinization, mixed with plasmid DNA and then immediately allowed to seed in the culture plates [28]).

For transfections with DNA-lipid complexes, Lipofectin reagent (Life Technologies) was used according to the manufacturer's recommendations and at a weight/weight ratio of 4 lipofectin/1 DNA, diluted in PBS and added (50 μl/0.5 μg DNA/well) to cultures in 96 well plates.

2.3.2. Adenovirus. Replication-defective virus was obtained as described [29,30]. It carries the firefly luciferase gene under the control of the Ad2 MLP promoter. Viral vector preparations (MOI ranging from 10 to 200 IU) were added to the cells cultured in 24 well plates in 100 μl PBS buffer. After 30 min incubation at 37°C in air plus 5% CO_2, 1 ml of culture medium was added in each well.

2.3.3. In vivo gene transfer. The luciferase plasmid (pTG11033 in TE buffer) was injected i.m. (25 μg/25 μl in both right and left tibialis anterior muscles) in 5–10 week old C57BL/10 mice, 4–6 mice per condition (total: up to 10 samples per condition). At various time points (1–8 weeks) after injection of the vectors, mice were killed and tibialis anterior muscles were retrieved and frozen. Luciferase activity was determined on the frozen muscle extracts. For glucocorticoid treatment, in order to avoid any interference, mice were administered i.m. on a daily basis in a different muscle (namely into the right and left quadriceps muscle alternately) than the plasmid-injected muscles. Treatment was 1 mg/kg (a commonly used dose) of α-methylprednisolone (Solumedrol), starting 2 days before plasmid injection, on a daily basis until 10 days after injection and 5 times per week through the 46 following days. Control animals were non-glucocorticoid-injected mice.

When performed, anesthesia was obtained after an intraperitoneal 100 μl injection of a mixture of 1 ml Imalgène 1000 (Rhône-Mérieux, Lyon, France), 0.5 ml Ronpun 2% (Bayer Pharma, Puteaux, France) and 5.2 ml NaCl 0.9%.

2.4. Parameters analyzed

Unless specified otherwise, cells were analyzed 48 h after transfection.

2.4.1. Reporter expression. Luciferase was extracted from cell layers or frozen muscle samples according to Manthorpe et al. [31]. Samples were analyzed using the Luciferase Assay System (Promega) in 96 well microtiter plates in a Berthold LB96P luminometer. For β-galactosidase (β-Gal) staining, cells were fixed with 2% formaldehyde/0.2% glutaraldehyde in PBS for 10 min at 4°C, and stained in a standard manner [32].

2.4.2. Detection of pTG11033 plasmid by Southern blot. DNA was extracted from the cultures and digested with *Hin*dIII (Life Technologies) according to manufacturer's specifications. Samples of 1 μg genomic DNA were loaded onto a 1% agarose gel. Various concentrations of plasmid were analyzed in parallel for quantitative estimation. After migration, denaturation and transfer overnight on Hybond-N$^+$ membrane (Amersham), hybridization was performed with a *Bam*HI-*Hin*dIII 700 bp fragment complementary to the luciferase expression cassette and radioactively labelled using the Megaprime DNA labelling system for autoradiography.

2.4.3. Protein concentration. Protein concentration was determined with the BCA protein assay kit (Pierce, Rockford, IL) using bovine serum albumin as standard.

2.4.4. Statistical analyses. Unpaired Student's *t*-test was used and the *P* value given.

3. Results and discussion

3.1. Dexamethasone enhances gene transfer in a concentration- and time-dependent fashion

Human myoblasts were cultured in the presence or absence of 10^{-6} M dexamethasone. Cells were transfected by the calcium phosphate transfection method with the luciferase reporter gene plasmid pTG11033, harvested 48 h later, and luciferase activity was determined. We observed that pretreatment of cell cultures for 24–48 h, and continuous presence of dexamethasone until cell harvesting, resulted in a significant increase in expression levels of the reporter gene (Fig. 1). When cells were transfected in suspension, the overall transgene expression and glucocorticoid enhancement were even greater (about 30-fold) than for adherent cells (two experiments, data not shown). Glucocorticoid treatment initiated at the time of (t_0) or 1, 4, or 24 h after transfection did not significantly increase reporter gene expression (Fig. 1A,B). The effect of dexamethasone on human myoblast transfection was concentration-dependent (no enhancement below 10^{-9} M, then increasing with a plateau at 10^{-7} M) (Fig. 1C). When the dexamethasone treatment was discontinued in pretreated cultures at the time of transfection, expression levels were lower than in continuously treated cultures (Fig. 1B). Transfection of human myoblasts with plasmids carrying a nls-*lacZ* expression cassette replacing the luciferase cassette on the same (pTG11034) or on a different plasmid backbone (pCH110N) showed more β-Gal-positive cells after dexamethasone treatment. While only very rare blue cells were seen 48 h following transfection (even of suspended cells), around 30% β-Gal-expressing myoblasts were obtained upon dexamethasone treatment (and after transfection of the resuspended human myoblasts). This suggests either that more cells are being transfected upon treatment, or a stimulation of expression above the detection threshold which would highlight more cells.

The transfection of both healthy (two donors) and DMD (one donor) human myoblasts was enhanced equally by dexa-

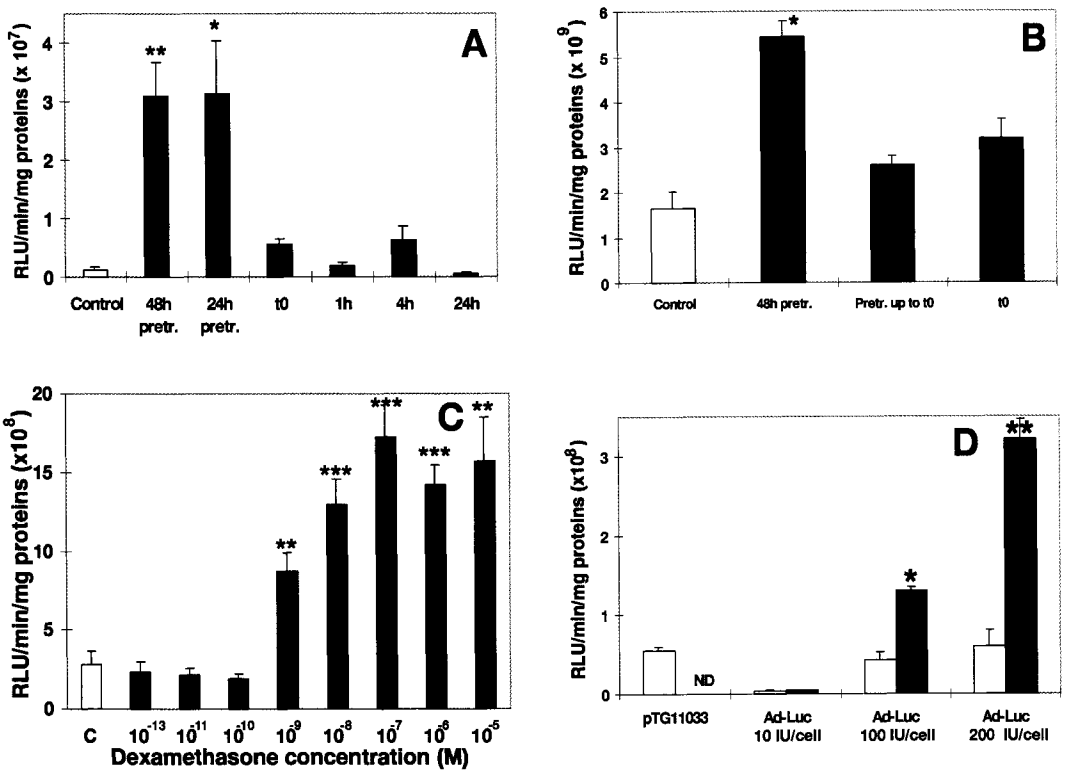


Fig. 1. Dexamethasone effect is dependent on concentration, onset and duration of treatment. A and B: Time dependence of dexamethasone effect. Calcium phosphate transfections performed on human myoblast cultures (donor Y). Empty bars: Control (non-dexamethasone-treated) transfected cultures. Full black bars: dexamethasone-treated cultures. 48h pretr., 24h pretr.: dexamethasone (1 μM) was added to the culture medium 48 h or 24 h prior to transfection and maintained throughout the culture. t0: dexamethasone added to the culture medium along with the calcium phosphate precipitated luciferase plasmid (pTG11033). 1h, 4h, 24h: onset of dexamethasone treatment at 1, 4, or 24 h after transfection, respectively. Pretr. up to t0: dexamethasone added 48 h before transfection and removed immediately prior to transfection. Luciferase activity was determined 48 h after transfection. Bars represent mean ± S.E.M. of four values of a particular experiment. The experiment was repeated twice and produced similar results. C: Concentration-response study of dexamethasone; effect on transfection of human (donor X) myoblasts with pTG11033 (luciferase plasmid). Not shown: cultures treated with ethanol (solvent for dexamethasone) at concentrations 0.01, 0.1 and 1% (the latter being 10 times in excess) with luciferase activities similar to non-glucocorticoid-treated cultures. Onset of treatment was 48 h prior to transfection and the treatment was maintained until cell harvesting (48 h post-transfection). Bars are mean ± S.E.M. of six values. The experiment was repeated once with similar results. D: Effect on transduction with an adenovirus luciferase vector (MLP promoter). Full black bars: dexamethasone (1 μM)-treated cultures (48 h pretreatment and maintained until harvesting, 48 h after gene transfer). ND: not determined. Bars are mean ± S.E.M. of six values. Myoblasts of donor X were used. $^{**}P < 0.01$; $^{*}P < 0.05$ as compared to control transfected cultures.

methasone (not shown), which excludes any artefact linked to a single cell type or cell clone. Considering the different muscles cultured (spinal, extensor digitorum longus, quadriceps), the dexamethasone effect does not seem to be linked to the type of muscle. Variations in amplitude of the glucocorticoid effect may be explained by several factors such as transfection efficiencies and responsiveness to glucocorticoids of primary cultured cells, and experimental conditions. An enhancement effect was also found in mouse primary myoblasts (85% increase, not shown), the human pulmonary cell line A549 (70% increase, not shown) and the human cell line 293 (97% ± 27% increase, seven experiments). On the other hand, primary dog myoblasts did not respond to the dexamethasone treatment, and VERO and CHO cells displayed only a mild (25%) dexamethasone-induced enhancement of transfection (data not shown). It remains unclear why some cells respond well to glucocorticoids whereas others do not or only moderately. Differences in constitutive glucocorticoid receptor expression or downstream mechanisms may be involved.

When compared to non-treated cells, at t_0, only a non-significant increase by 13.0 ± 12.9% (six experiments) of total protein content and by 10.8 ± 3.8% of the cell number (counted after cell dissociation) was observed in dexamethasone-treated cells. At the time of cell harvest, a slight increase (29.1 ± 12.3%, 10 experiments) of total proteins was observed in glucocorticoid-treated cells. Compared to this small increase in total proteins, the increase in expression levels was much higher. Furthermore, dexamethasone-induced increase of luciferase expression was also found in mitomycin C-arrested cells. Mitomycin (5 μM) was added to the culture medium of dexamethasone-treated cells or non-glucocorticoid-treated cells, 24 h before transfection with luciferase plasmid (pTG11033). Prolonged exposure (48 h) to mitomycin was toxic. Cultures were rinsed 4 h after transfection, and mitomycin was then omitted, whereas dexamethasone treatment was maintained throughout the experiment. Dexamethasone increased luciferase activity 4.4-fold in mitomycin-arrested cultures ($4.27 \pm 0.82 \times 10^8$ versus $1.04 \pm 0.21 \times 10^8$ RLU/mg proteins, $P < 0.01$) and 4.8-fold in the same experiment in non-arrested cultures ($n = 6$, $P < 0.001$) (two experiments performed). This suggests that the enhanced expression in glucocorticoid-treated cell cultures cannot simply be explained by

an increase in cell number subjected to transfection or by their higher survival after transfection. Luciferase activity was two-fold higher in the non-mitomycin-treated cultures as compared to the mitomycin-arrested cultures ($P < 0.05$), which could be explained by the overall toxicity of mitomycin and/or the lower transfection capacity of the non-dividing cells.

3.2. The observed effect is specific to glucocorticoids

The two other glucocorticoids tested, cortisol and α-methylprednisolone (both at 10^{-6} M and with 48 h pretreatment) enhanced luciferase activity as did dexamethasone. Their respective induced increases of transgene expression were 3.3, 3.3 and 2.4 with P values < 0.001, 0.001 and 0.05 respectively ($n = 4$ wells). Other steroids (progesterone and testosterone) or the β-adrenergic agonist anabolic clenbuterol did not influence transgene expression (not shown), whereas the enhancing effect was found constantly throughout the 20 experiments involving glucocorticoids.

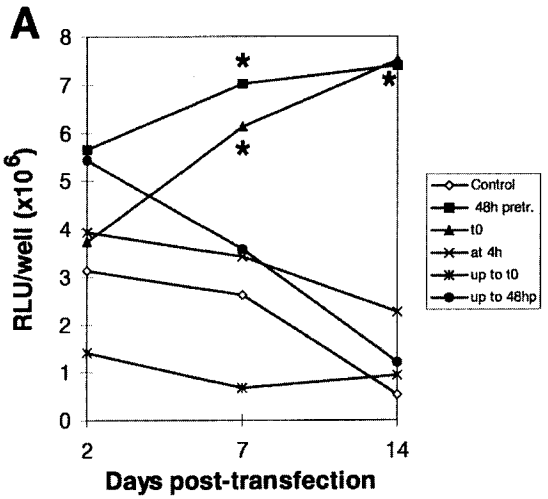
3.3. The effect was not linked to the protocol of transfection nor to the promoter sequences or reporter gene

The efficiency of lipofection with our luciferase plasmid pTG11033 complexed with lipofectin was also increased by dexamethasone; luciferase activity in transfected cultures was respectively $4.1 \pm 1.7 \times 10^7$ RLU/mg proteins and $18.6 \pm 5.2 \times 10^7$ RLU/mg proteins ($P < 0.001$, $n = 6$ wells). Transduction with an adenovirus encoding the luciferase gene under control of the MLP promoter was also enhanced by dexamethasone (Fig. 1D). The lack of influence of the nature of the promoter was further demonstrated by the similar dexamethasone-induced enhancements of transfection with plasmid pTG11174 containing the RSV promoter instead of the CMV promoter (but otherwise identical to our luciferase plasmid pTG11033) with respective luciferase activities being $1.2 \pm 0.5 \times 10^7$ and $11.4 \pm 3.5 \times 10^7$ RLU/mg proteins ($P < 0.001$, $n = 6$ wells), as well as with the β-Gal plasmid which contains the SV40 early promoter.

3.4. Binding of glucocorticoid to the cytoplasmic receptor and the role of GRE sequences

RU38486, the steroid receptor antagonist, was added (10 μM) to the culture medium 1 h before dexamethasone (1 μM), that is 48 h prior to transfection. Both were maintained until cell harvesting (48 h after transfection). RU38486 abolished the dexamethasone effect: there was no significant difference in luciferase activity between dexamethasone+RU38486-treated cultures ($9.8 \pm 2.0 \times 10^8$ RLU/mg proteins) and cultures treated with RU38486 alone ($6.7 \pm 1.0 \times 10^8$ RLU/mg proteins), whereas in the same experiment $54.4 \pm 3.4 \times 10^8$ RLU/mg proteins where found in the dexamethasone-treated cultures and $16.5 \pm 3.6 \times 10^8$ RLU/mg proteins in control untreated cultures ($P < 0.01$; $n = 6$ wells). This shows that binding of the glucocorticoid to its receptor is necessary. The slight decrease in luciferase activity found in RU38486-treated cultures as compared to control could be due to inhibition of cortisol present in low amounts (10^{-8}–10^{-9}) in the fetal bovine serum used in the culture medium.

On the other hand, we found that the presence of the *cis*-regulatory DNA GRE sequences [13] on the plasmid were not required for the glucocorticoid effect, since transfection with a plasmid that did not contain GREs was also increased by dexamethasone. Comparisons were performed with two isogenic plasmids containing the same promoter plus (pTG11096) or minus (pTG11056) GRE sequences. Dexamethasone (48 h pretreatment) stimulation of human myoblast transfection was obtained with both plasmids. In this experi-



B

Estimated number of plasmid copies per culture

Time after transfection	4hrs	2 d	7d	14d
-	11840	460	390	400
+	37110	1420	1940	1830

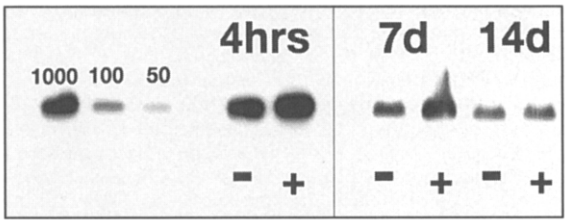


Fig. 2. Short and long term effects of dexamethasone. A: Luciferase activity of cultures transfected with calcium phosphate-precipitated pTG11033 measured 2, 7 or 14 days after transfection. Control (non-dexamethasone-treated) cultures (empty squares); 48h pretr.: cultures treated with dexamethasone starting 48 h before transfection and maintained throughout the experiments (black squares); t0: dexamethasone treatment starting at the time of transfection and corticoid maintained thereafter (black triangles); at 4h: dexamethasone added to the culture medium 4 h after transfection and maintained thereafter (crosses); up to t0: dexamethasone treatment beginning 48 h prior to transfection and omitted thereafter (stars); up to 48hp: dexamethasone treatment beginning 48 h prior to transfection and removed from the culture medium 2 days after transfection. Values are mean of six wells. $^*P < 0.05$. B: Plasmid detection in untreated (−) and dexamethasone-treated (+) cell cultures transfected with pTG11033. Cells (in 60 mm Petri dishes, one or two dishes per time point) were treated with 10^6 M dexamethasone starting 2 days before transfection and treatment was maintained throughout the experiment (up to 14 days after transfection). Lower panel: Southern blot of pTG11033. Cells were arrested 4 h after transfection (4hrs) or 2 (2d), 7 (7d) or 14 (14d) days after transfection. 1000, 500, 50 are pTG11033 plasmid copies. Data are from one experiment. A similar pattern was obtained in a second experiment.

ment (repeated once, with myoblasts of donor X and of donor Y), luciferase activities in control cultures transfected with pCMV plasmid (pTG11033), pTG11096 and pTG11056 were respectively 7.5 ± 1.3, 8.2 ± 1.2 and $5.8 \pm 0.6 \times 10^8$ RLU/mg proteins and were increased respectively 7.0-, 4.5-, and 8.0-fold upon dexamethasone (1 μM) treatment ($n = 6$; P values all < 0.001).

This suggests that the enhancement of reporter gene expression is not a direct response but rather mediated by cellular GRE-responsive gene(s). This interpretation is in contradiction with, but does not exclude, the possibility stated elsewhere [33] of a plasmid nuclear translocation driven by cytoplasmic glucocorticoid receptor bound to plasmid GREs. The effect described here may not occur through a classical GRE-mediated mechanism, which has been reported previously in other cases (see for example [34]).

3.5. *Intracellular calcium and arachidonic acid cascade are not involved in the dexamethasone effect on cell transfection*

The anti-inflammatory effect of glucocorticoids is attributed to a significant degree to their suppressive activity on the arachidonic cascade at multiple levels [13]. We investigated this pathway using different enzyme inhibitors. The molecules tested, quinacrine 30 μM, nordihydroguaiaretic acid 0.01 μM and indomethacin 0.1 μM, which inhibit phospholipase A2 (as do glucocorticoids [19]), cyclooxygenase and lipoxygenase, were without effect on transfection of human myoblasts (the test molecules were added to the culture medium 48 h before transfection and maintained until cell harvesting 2 days after transfection).

As calcium metabolism may also mediate glucocorticoid effects [13] we investigated methoxyverapamil or the calcium ionophore ionomycin (both at 1 μM) which either decrease or increase intracellular calcium concentration. Both agents had no significant influence on the dexamethasone effect on human myoblast transfection. Luciferase activities of non-treated control, methoxyverapamil, and ionomycin cultures were 4.1 ± 1.5, 3.7 ± 0.3 and $1.9 \pm 0.4 \times 10^8$ RLU/mg proteins respectively and 13.7 ± 0.8, 17.2 ± 1.6 and $4.9 \pm 0.5 \times 10^8$ RLU/mg proteins in their dexamethasone-treated counterparts (P values all < 0.01, $n = 6$). The lower luciferase activity observed in ionomycin-treated cultures could be explained by toxicity provoked by the elevated cellular calcium content over the 4 day duration of the treatment.

As pretreatment of at least 24 h with glucocorticoids produced a maximal effect on cell transfection, one can speculate that an accumulation of factor(s) may be necessary. Since the effect was not dependent on transfection protocols and since adenoviral vectors and plasmids do not use the same penetration mechanisms, our observations may reflect at least in part an accumulation of the factor(s) inside the cell. Whether this accumulation is due to increased transgene expression, increased messenger or protein stability remains to be investigated. This observation also excludes direct short term effects of glucocorticoids such as increased membrane fluidity [18] (which would enhance vector penetration into cells), or short term effects on vector DNA transcriptional or post-transcriptional processes. Therefore the most likely mechanism of action might be an indirect one, through the regulation of cellular (i.e. transcriptional) factors, but with no implication of the arachidonic cascade and changes in cytoplasmic calcium.

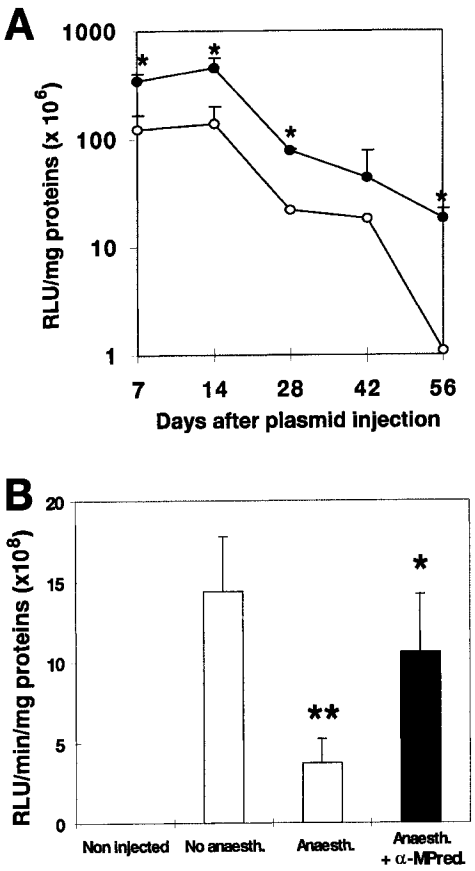


Fig. 3. Glucocorticoid increases transgene (luciferase) expression in vivo. A: Kinetics of luciferase activity of tibialis anterior muscles 7 days following pTG11033 injection in non-anesthetized control (empty circles) or α-MPred-treated mice (black circles). α-Methylprednisolone (a-MPred) was diluted in NaCl 0.9% and injected at a final dose of 1 mg/kg. Symbols are mean ± S.E.M. of eight values. The experiment was repeated twice with similar results. B: Injection of pTG11033 plasmid was performed in control anesthetized (Anaesth.) or in α-MPred-treated anesthetized mice (Anaesth.+α-MPred). No anaesth.: non-anesthetized mice injected with pTG11033. The injected muscles (right and left tibialis anterior) were retrieved 7 days after plasmid injection. Bars are mean ± S.E.M. of eight values. Bars are mean ± S.E.M. of 10 values. $^*P < 0.05$ as compared to Anaesth.; $^{**}P < 0.01$ as compared to No anaesth. The experiment was repeated four times with similar results.

3.6. *Dexamethasone increases reporter gene expression long term*

As shown in Fig. 2A, in the absence of dexamethasone, luciferase activity decreased with time (empty squares) (end of experiment: 14 days after transfection). This drop was not observed in dexamethasone-treated cultures (dexamethasone applied 48 h before transfection and maintained throughout the culture, full black squares). As also shown above, no difference in luciferase activity was observed 2 days after transfection in cultures treated with dexamethasone at t_0 of transfection (full black triangles) as compared to non-treated cultures (empty squares); however, at later time points, as dexamethasone was maintained in the culture medium, luciferase expression increased (full black triangles). When dexamethasone treatment was applied 4 h after transfection, no long-term increase was obtained. When dexamethasone was applied up to t_0, no increase (short or long

term) was found (double crosses). Upon dexamethasone removal from the culture medium 48 h after transfection of glucocorticoid-pretreated cultures, transgene expression dropped (full black circles). Differences at 14 days post-transfection between t_0 dexamethasone-treated cultures and 48 h pretreated cultures may have been attenuated over the long term or may reflect a maximum of transgene expression. A significant expansion in cell number was observed over the 16 days of this experiment, which would dilute out the expression from our non-replicating plasmid, thus leading to underestimation of the dexamethasone effect. Therefore, the results presented are not being expressed per mg proteins.

3.7. *Effect of dexamethasone on plasmid entry and maintenance into cells*

As shown in Fig. 2B, the amount of transfected plasmid was increased approximately 3 times in dexamethasone-treated cultures at 4 h post-transfection. Nevertheless, at this time point results may have been biased by the plasmid DNA which may have been retained on the cellular membrane. This possibility is, however, unlikely for the further time points since untransfected plasmid DNA should be degraded shortly after incubation with the cultures.

We found that plasmid signal was also increased in dexamethasone-treated cultures 2 days (3.1 times), 7 days (5 times) and 14 days (4.6 times) after transfection. The number of plasmid copies per culture remained stable between 2 and 14 days after transfection in both control and dexamethasone-treated cultures (two experiments).

Therefore, two major effects are found: (A) a transient increase of gene expression of glucocorticoid-pretreated cells through increase of plasmid entry and perhaps through transgene expression and (B) a long term enhancement of gene expression through an action on the level of transgene expression or downstream mechanisms.

3.8. *Glucocorticoid increase i.m. gene transfer in vivo*

C57BL/10 mice were treated with α-methylprednisolone (1 mg/kg, i.m. – quadriceps – injections) on a daily basis, starting 2 days before plasmid (pTG11033) injection into right and left tibialis anterior. Luciferase activity of each of the whole muscles was determined at various time points after plasmid injection (which corresponds to the peak time point of transgene expression, according to our previous experiments, not shown). We found that luciferase activity was increased 3 times in α-methylprednisolone-treated animals (Fig. 3A) 7 days after injection. Luciferase activity decreased starting 2 weeks after injection in both control and α-methylprednisolone-treated mice. We observed that, when mice were anesthetized (with an intraperitoneal injection of either xylazine/ketamine or pentobarbital) before plasmid injection, transgene (luciferase) expression was lowered (by a factor of 4) as shown in Fig. 3B (only results of xylazine/ketamine-anesthetized mice are shown). This phenomenon measured 7 days after plasmid administration was partially reversed due to the α-methylprednisolone effect (Fig. 3B).

Glucocorticoids may potentially increase in vivo gene transfer per se, but an indirect effect through different mechanisms such as anti-inflammatory [17] and immunosuppressive actions cannot be excluded. However, the long term effect was not confirmed in vivo. Additional investigations are needed to study more precisely both phenomena. The explanation for the impairment of gene transfer due to anesthesia carried for i.m. plasmid injection also deserves more specific investigations. It could be related to the lack of muscle contractile activity during anesthesia (which lasted about up to 20 min after plasmid injection), which could lead to impaired diffusion of the vector into the muscle tissue or lack of charge repulsions between the resting muscle fibers (which are negatively charged in contrast to contracting muscle fibers) and the negatively charged plasmid DNA.

In conclusion, the present report demonstrates that it is possible to pharmacologically enhance gene transfer into skeletal muscle cells. This may lead to important applications in ex vivo and direct in vivo delivery approaches of therapeutic genes in patients with neuromuscular disorders.

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