Cortisol increases transfection efficiency of cells

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Abstract DNA uptake can be facilitated by addition of physiological amounts of 11β-hydroxy glucocorticosteroids (such as cortisol) during transfection. In the presence of cortisol, but not of the inactive 11-keto glucocorticoid cortisone, twice as many cells uptake and express the reporter gene. The effect is specific and dose-dependent; the amounts of glucocorticosteroids needed to enhance transfection efficiency are in the nanomolar range, which corresponds to the dissociation constant of glucocorticoids for the glucocorticoid receptor in vitro. This effect can be abolished by an excess of the glucocorticoid antagonist RU486. We infer that the activated cytoplasmic glucocorticoid receptors enhance nuclear translocation of the incoming transfected DNA.

Key words: 11β-glucocorticosteroid; Glucocorticoid receptor; Gene transfer

1. Introduction

The use of gene therapy to treat genetic diseases has increasingly become the focus of research. The ideal delivery system should have at least the following characteristics: the ability to target cells effectively, the ability to transduce a large number of cells regardless of their mitotic status, and the potential to be synthetic and of low toxicity. Several delivery systems have been designed based on either recombinant viruses or non-viral vectors [1]. A recurrent problem, however, is the relative poor efficiency of nuclear translocation of the foreign DNA from the cytoplasm to the nucleus [2,3]. Even if the efficiency with which the DNA-vehicle formulation crosses the cellular membrane is relatively high, and even if the release of the exogene complex from the endosome is efficient, only a fraction of the DNA actually reaches the nucleus. One way to solve this problem is to include in the delivery system peptides that contain a nuclear localisation signal [4–6]. This approach has advantages and disadvantages. The obvious advantage is a much improved nuclear translocation. The major disadvantage is the relative lack of versatility. Having to introduce a nuclear peptide in the delivery system may severely limit the choice of the vehicle and may involve complicated chemical linkage steps. In turn, this can limit the choice of delivery systems that ensure target specificity, which is usually related to the type of vehicle used [7]. With this regard, the use of a nuclear translocation system that does not influence the versatility of vehicles and at the same time can be used in a wide variety of cells would be extremely advantageous.

We asked whether cytoplasmic steroid receptors could improve the efficiency of transfection. Cytoplasmic glucocorticoid receptors become activated by incoming 11β-hydroxy glucocorticosteroids, translocate to the nucleus and bind DNA sequence specifically [8–10]. We reasoned that if glucocorticoid receptors can be activated and meet a transfected DNA directly in the cytoplasm, they might bind the exogene (which if long enough will contain glucocorticoid-binding sequences) and carry it to the nucleus, thus improving transfection efficiency. Moreover, glucocorticoid receptors are ubiquitous, and a positive result in this direction may provide a useful tool for improving transfection efficiency of a wide variety of cells.

2. Material and methods

2.1. Cell culture

3Y1 cells [11] and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS (PAA Laboratories, Linz, Austria) at 37°C under 5% CO_2.

2.2. Glucocorticoid receptor-binding assays

For binding assays, cells were harvested and lysed in a hypotonic buffer (10 mM Tris pH 7.5/10 mM NaCl/1 mM EDTA/10% glycerol/10 mM sodium molybdate/10 mM β-mercaptoethanol) in a dounce homogeniser (B-pestel). The cell extract was centrifuged at $10000 \times g$ for 1 h, and the protein concentration adjusted to 0.6 mg/ml. One hundred μl (60 μg, corresponding to the extract about 2×10^6 cells) were used for each sample. The samples were incubated in the same buffer but with 100 nM NaCl in presence of 10 pmoles [1,2,4-^{3}H]dexamethasone (2×10^{17} dpm/mole) and increasing amounts of unlabelled steroid competitors (1–1000-fold excess) on ice for 2 h. The reaction was stopped by addition of active charcoal to a final concentration of 20 mg/ml to absorb excess, unbound steroids. The supernatant was spotted onto glass microfibre filters (Whatman 1822-025) and washed extensively in 20 mM Tris pH 8, 1 mM EDTA, 40 mM NaCl. The filters were patted dry, introduced in counting vials with Irga-safe-plus scintillation fluid (Packard) and counted in a Packard beta-counter.

2.3. Plasmids, transfection protocol, and X-gal staining

The CMV-LacZ reporter plasmid consists of the bacterial β-galactosidase gene under control of the cytomegalovirus promoter in a pUC18 background. For transfection, 5 μg CMV-lacZ plasmid DNA were mixed with 20 pmoles cortisol, 20 pmoles cortisone or an equivalent amount of solvent (ethanol) in 500 μl TBS. For competition experiments, 20 nM cortisol was used with the addition of 20 μM RU486, a glucocorticoid antagonist.

After incubation at room temperature for 15 min, 500 μl of a solution of 10 μg/ml polyethyleneimine (PEI) in TBS was added dropwise to the DNA/steroid mixture. This PEI solution was prepared by diluting 100 μl PEI Stock in 10 ml TBS, where the stock consists of 90 mg PEI (50% aq. sol., 50000 kDa, Sigma) in 50 ml H_2O [12]. The DNA-steroid-PEI complex (1 ml, final steroid concentration 20 nM) was overlaid directly onto 3Y1 cells (in 6 cm dishes, 50% confluent) which had been rinsed twice with TBS. The cells were then incubated at room temperature for 3–4 h. The mixture was then removed, and 3 ml of fresh medium (without steroids) was added.

After a 3 day culture, the cells were washed twice with cold PBS supplemented with 2 mM $MgCl_2$ and fixed in ice-cold PBS/2 mM $MgCl_2$/2% formaldehyde/0.5% glutaraldehyde at 4°C for 15 min.

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The cells were subsequently rinsed with PBS/2 mM $MgCl_2$ and stained with PBS/2 mM $MgCl_2$/0.05% NP40/5 mM $K_3[Fe^{II}(CN)_6]$/5 mM $K_4[Fe^{III}(CN)_6]$. A fixed area of the dishes was then examined microscopically, and the number of blue cells was determined.

3. Results and discussion

3.1. Choice of model system

To test whether 11β-glucocorticosteroids can enhance through the glucocorticoid receptor (GR) the efficiency of transfection, we first looked for an appropriate model system. In the ideal system, the number of incognita would have to be minimised, in particular it should consist of cells that express reasonable levels of glucocorticoid receptor. Only thus an inequivocable proof of principle can be obtained, and the model can be subsequently extended to other cell types.

Normally, mammalian cell extracts do not contain enough glucocorticoid receptor to yield any measurable binding of radiolabelled glucocorticoids, unless the cells are transfected with a GR expression vector. In contrast, rat 3Y1 cells [11] contain levels of GR high enough to yield significant and specific binding. As shown in Fig. 1, 60 μg of 3Y1 cell extracts (the equivalent of about 2×10^6 cells) reproducibly bind about 50 fmoles of [1,2,4-^{3}H]dexamethasone (2×10^{17} dpm/mole). From these data, we calculated that 3Y1 contain about 20000 GRs per cell. The binding is specific, because it can be competed efficiently by an excess of non-labelled dexamethasone. Less active steroids can do so only less efficiently; for instance, the inhibition constant (IC50) of cortisol-21-hemisuccinate is 10-fold higher than that of cortisol (data not shown).

3.2. 11β-glucocorticoids enhance transfection efficiency of 3Y1 cells

We tested the effect of steroids on the transfection efficiency of 3Y1 cells using a reporter plasmid, CMV-LacZ, which carries the bacterial β-galactosidase gene under the control of the human cytomegalovirus promoter. Five μg DNA were transfected by the polyethyleneimine method [12] or the calcium phosphate method to 3Y1 cells in 6 cm dishes in the absence of steroids, with 20 nM cortisone or 20 nM cortisol (the active 11β-OH form). Cortisone is an 11-keto compound; it cannot bind the glucocorticoid receptor and is therefore inactive. Cortisol, in contrast, is an 11β-hydroxy compound and has full steroid activity.

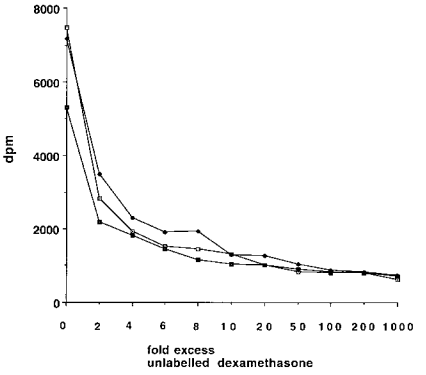

Fig. 1. Estimate of GR content in 3Y1 cells based on specific binding of [^{3}H]dexamethasone. Sixty μg of 3Y1 cell extracts, corresponding to the extract of about 2×10^6 cells, were incubated with 10 pmoles [1,2,4-^{3}H]dexamethasone (2×10^{17} dpm/mole) and increasing amounts of unlabelled steroid competitors (1–1000-fold molar excess), and binding activity was determined as described in Section 2. Three independent determinations are displayed.

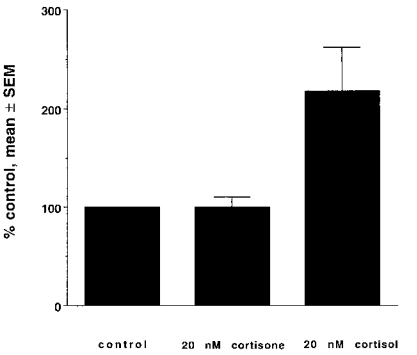

Fig. 2. Cortisol enhances transfection efficiency of 3Y1 cells. Five μg CMV-lacZ plasmid DNA were mixed with 20 pmoles cortisol, 20 pmoles cortisone or an equivalent amount of solvent (ethanol) and transfected to 3Y1 cells by the polyethileneimine method (PEI) [12] (see Section 2). We also performed experiments with the calcium phosphate method to verify that the results are reproducible under various conditions. Transfection efficiency of each sample was represented as the percent of control. The results shown in this figure represent the mean ± S.E.M. from six independent experiments, of which one was carried out with the calcium phosphate method (with similar results).

If cortisol influences transfection in a positive way, more cells should express the reporter gene in its presence. However, this effect, if observed, may be explained in several ways. On one hand, it is possible that steroids do not influence how much exogene is internalised and transferred to the nucleus. Similar amounts of the exogene may reach the nucleus of 3Y1 cells, but only in those cells exposed to cortisol transcription may be unspecifically enhanced, so that the threshold of expression of the transfected β-galactosidase may be more easily reached. This hypothesis is nominally possible, but is unlikely. First, there are no reports that glucocorticoids enhance transcription unspecifically. Second, the CMV promoter is constitutive, not glucocorticoid-dependent and very strong [13–15]. On the other hand, it is possible that glucocorticoids improve the cell's capability of endocytosis. However, endocytosis of the exogene takes place within 3 h from exposure to the DNA-vehicle complex, and small amounts of steroids cannot influence cellular metabolism by GR-mediated events quickly enough to bear any influence on this process. To avoid these potential problems, we incubated the cells with cortisol only during the 4 h exposure to DNA, and removed it from the cells right after the transfection by rinsing extensively with PBS and adding fresh medium. Exposure of cells to cortisol for only 4 h does not result in any measurable glucocorticoid-dependent gene transcription. In fact, exposure of Kiki cells [16] to dexamethasone for only 4 h is insufficient to induce any measurable glucocorticoid-dependent expression of β-galactosidase (data not shown). Kiki cells are a permanent cell line derived from 3Y1 cells, and carry the lacZ reporter gene under the control of the mouse mammary tumour virus (MMTV) promoter, a strong promoter that contains essential glucocorticoid-responsive elements [17–19]. Under these conditions, steroids must thus exert their effect on what happens to the exogene only after internalisation. If more cells can be transfected, it is very likely that the activated glucocorticoid receptor facilitates or accelerates nuclear transport.

The percentage of cells expressing β-galactosidase was assessed microscopically after fixation and staining with X-gal. As shown in Fig. 2, the presence of cortisol increases the number of transfected cells at least 2-fold. The effect is not due to an alteration in the cell membrane's properties in presence of a lipophilic agent like a steroid, because cortisone does not improve transfection. The increased transfection efficiency is therefore specifically dependent on steroid activity. For the reasons given above, the increase in the number of cells that express the exogene is not due to unspecific enhancement of transcription. Taken together, these data imply that in the presence of an active 11β-hydroxy glucocorticoid, more cells become competent to transfer transfected DNA into their nucleus.

The positive effect of cortisol on transfection efficiency is dose-dependent (Fig. 3). The window of active concentrations is unusually narrow, starting with 1 nM and reaching a plateau with 2 nM, which is in the range of the dissociation constant of glucocorticoids with the glucocorticoid receptor [20–23]. Higher concentrations of steroids do not further improve transfection efficiency. In addition, the glucocorticoid antagonist RU486 reduces the effect of cortisol (Fig. 4).

3.3. 11β-glucocorticoids enhance transfection efficiency of HeLa cells through the GR

The question that remained to be answered was, does cortisol increase the efficiency of transfection in cells other than rat 3Y1? We tested the effect of cortisol, in presence and absence of the antagonist RU486, on rat 3Y1 cells and human HeLa cells. The latter do not express high levels of glucocorticoid receptors, and in this regard are representative of more common cell lines. As shown in Fig. 4, cortisol enhances the transfection efficiency of HeLa cells as well as 3Y1 cells, and this effect is reduced by the addition of RU486.

3.4. Conclusion

In conclusion, we have observed that physiological concentrations of cortisol, administered to the cells during transfection, increases the chances of effective DNA uptake in rat 3Y1 cells and human HeLa cells. The effect is dependent on steroid activity and is probably due to increased nuclear translocation of the transfected DNA. Because glucocorticoid receptors are essentially ubiquitous, both with regard to tissue distribution and species, this observation is important for all those who wish to improve DNA delivery to cells.

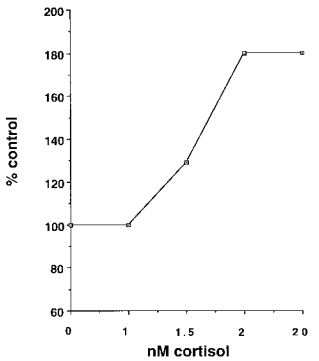

Fig. 3. Dose dependency of cortisol. Increasing amounts of cortisol in the nM range were incubated with 5 μg CMV-lacZ plasmid DNA and transfected to 3Y1 cells by the polyethyleneimine method. Transfection efficiency of each sample is represented as the percent of control.

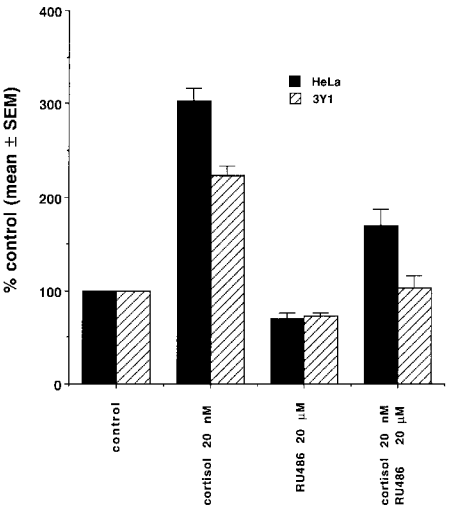

Fig. 4. Cortisol enhances transfection efficiency of 3Y1 and HeLa cells through the glucocorticoid receptor. Five μg of CMV-lacZ were incubated with no addition, 20 nM cortisol, or 20 nM cortisol + 20 μM RU486 and transfected to 3Y1 and HeLa cells by the PEI method. Transfection efficiency of each sample is represented as the percent of control. The results represent the mean ± S.E.M. ($n = 5$).

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