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Macromolecular synthesis inhibitors perturb glucocorticoid receptor trafficking

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ABSTRACT

The ability of inhibitors of transcription and translation to prevent glucocorticoid-induced apoptosis has been interpreted to indicate that the cell death machinery requires *de novo* protein synthesis. The transcriptional inhibitors actinomycin D (Act D) and DRB as well as the translational inhibitors CHX and puromycin inhibited early loss of mitochondrial membrane integrity in a dose-dependent manner. This effect was not observed with the transcriptional inhibitor α-amanitin suggesting they may have additional effects. Their role in the glucocorticoid receptor (GR) intracellular trafficking was therefore investigated. Here, we show that Act D and CHX reduced glucocorticoid binding, GR turnover and impaired GR nuclear translocation. We performed the same experiments in different thymocyte subpopulations of Balb/c mice. At the highest dose tested, actinomycin D and cycloheximide abolished glucocorticoid-induced cell death of CD4+CD8+ and CD4+CD8−. In all subsets, Act D, DRB, as well as CHX and puromycin prevented receptor nuclear translocation, indicating a general alteration of GR trafficking. Overall, our data support a direct effect of macromolecular inhibitors on GR activation and trafficking. Finally, direct alterations of the functional properties of the glucocorticoid receptor might be responsible for cell death prevention by actinomycin D, DRB, cycloheximide and puromycin.

1. Introduction

Glucocorticoid-induced apoptosis of thymocytes was one of the first described programmed cell death [1] but mechanisms by which glucocorticoids (GC) elicit cell death are still unresolved [2]. At the molecular level, GC binds to the glucocorticoid receptor (GR), a member of the nuclear receptor family. GR is primarily located in the cytoplasm as large complexes with heat shock proteins and chaperones which are required for proper steroid binding [3]. Hormone binding triggers GR translocation to the nucleus where the GC/GR complex transactivates or represses gene expression through glucocorticoid response elements (GRE) [4]. Transrepression results from direct or indirect interference with several transcription factors such as NF-κB and AP-1 at both the cytoplasmic and nuclear levels [5]. GC also have rapid non-genomic effects, observed within seconds or minutes [6]. These effects are too fast to be regulated by transcriptional and translational steps. They involve generation of second messengers (Ca++, IP3, DAG or AMPc) and GR-interacting protein kinases and phosphatases [6–8].

GC-induced cell death involves cascades of events, including mitochondrial permeability transition, cytochrome c release, caspase activation and DNA fragmentation [2]. However, initiating events are still a matter of controversy. In several studies, *de novo* RNA and protein synthesis have been found to be required since actinomycin D (Act D) and cycloheximide (CHX), respective inhibitors of transcription and translation, prevent dexamethasone-induced apoptosis [9]. This scheme seems further validated by recombinant mice expressing a mutated GR devoid of dimerization property (GR^{dim}) that retains transrepressive but no transactivating and apoptotic activities [10]. This work validates the model by which GC induce the expression of "death genes" although such genes have not been found at the moment [2,9]. On the other hand, results obtained with GR mutants displaying reduced transactivation activity support the idea that GC-induced apoptosis *in vitro* is restricted to the transrepressive activity of the glucocorticoid receptor on "survival genes" [11,12].

This scheme is rendered even more complex by the use of mouse thymocytes, a widely used physiological model of glucocorticoid-induced cell death due to its extreme sensibility to corticoids. The thymus provides an inductive environment for development of T-lymphocytes from hematopoietic progenitor cells, which are

Abbreviations: Act D, actinomycin D; CHX, cycloheximide; Dex, dexamethasone; DN, double negative cells; DP, double positive cells; DRB, 5,6-dichlorobenzimidazole 1-β-D-ribofuranoside; GC, glucocorticoid; GR, glucocorticoid receptor; GRE, glucocorticoid response element; SP, single positive cells; $\Delta\Psi_m$, mitochondrial membrane potential.

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critical cells of the adaptive immune system. Thymocytes are divided in four subsets, the single positive (SP) CD4+ or CD8+ cells, the double negative (DN) CD4−CD8− and double positive (DP) CD4+CD8+ cells. Significant differences of GR expression were observed in these subsets [13]. The highest GR expression was found in DN thymocytes, and decreased during development via the CD8+ subpopulation into the DP subset [13,14]. Interestingly, the latter population, although expressing the lowest GR level was the most sensitive to GC-induced apoptosis [13], raising the possibility that apoptosis may not be induced by transcription regulation but by non-genomic pathways [15]. Additionally, mitochondrial GR translocation in this DP population was found to correlate with their sensitivity to glucocorticoid-induced apoptosis [16]. Taken together, these data indicate that the exact role of the glucocorticoid receptor and the subsequent activation of the apoptotic pathway are far from being fully elucidated and may also depend on the cellular context.

The assumption that glucocorticoid-induced apoptosis requires *de novo* protein synthesis has been previously re-evaluated in thymocytes, the most physiological model [17]. Studies with rat thymocytes indicated that DNA cleavage induced by thapsigargin, methylprednisolone, a synthetic glucocorticoid, and ionomycin were inhibited by cycloheximide and emetine but not by puromycin at concentrations that reduced protein synthesis by more than 80% [17], demonstrating that the degree of translational blockade induced by these inhibitors did not correlate with their protective effect and suggesting they could delay the onset of apoptosis rather than prevent it. Interestingly, drugs known to interfere with GR macromolecular complexes stability, such as geldanamycin, alter GR nuclear translocation, transactivation and transrepression activities and also inhibit glucocorticoid-induced apoptosis [18–21]. Taken together, these data suggested cycloheximide and actinomycin D might affect glucocorticoid receptor trafficking, thus preventing cell death. Therefore, cellular localization of glucocorticoid receptor in all thymocyte subsets, free or steroid-bound was carried out in the presence or the absence of macromolecular inhibitors: cycloheximide, DRB, actinomycin D or puromycin. Our data suggest that alteration of the functional properties of the glucocorticoid receptor, rather than inhibition of GR-induced death genes expression, might be responsible for cell death prevention by macromolecular inhibitors and indicate that the exact role of these inhibitory drugs is far more complex than initially conceived and depends on the cellular context.

2. Materials and methods

2.1. Reagents

Non-radioactive dexamethasone, cycloheximide, alpha-amanitin, 5,6-dichlorobenzimidazole 1-β-D-ribofuranoside (DRB), puromycin dihydrochloride were purchased from Sigma. Actinomycin D was purchased from Calbiochem. [6,7-3H]-Dexamethasone ([^{3}H]-Dex; 35–50 Ci/mmol) was purchased from Perkin Elmer (Zaventem, Belgium). Stock solutions of alpha-amanitin and puromycin were made in H_2O while dexamethasone, DRB, Act D and CHX were prepared in ethanol.

2.2. Thymocytes and splenocytes preparation and culture

Thymocytes were prepared from 5 to 8 weeks old Balb/c mice as described [22]. Cells were cultured at 10×10^6 cells/ml in RPMI medium without phenol red (Gibco BRL), supplemented with 10% heat-inactivated and charcoal-stripped foetal calf serum, 1000 UI/ml penicillin, 1000 μg/ml streptomycin and incubated at 37 °C in 5% CO_2. Dexamethasone was used at 10^{-7} M for 1 or 4 h (see details in the legends).

2.3. Flow cytometry analysis

Mitochondrial membrane potential and plasma membrane permeabilisation were assessed by flow cytometry analysis using CMXRos fluorescent dye and the impermeant DNA intercalatant YOPRO-1, respectively (Molecular Probes) [23,24]. Briefly, cells (5×10^5) were incubated with CMXRos (50 nM) and YOPRO-1 (200 nM) for 30 min in culture medium at room temperature in the dark and analyzed by flow cytometry (EPICS XL or CYAN, Beckman Coulter) using Expo 2 or Summit analysis softwares. High CMXRos and low YOPRO-1 cells were considered as living cells whereas low CMXRos and high YOPRO-1 cells were considered as apoptotic cells.

Immunophenotyping was carried out using the CYAN apparatus (Beckman Coulter), using PE rat anti mouse CD4 (IgG2b, κ) and APC rat anti mouse CD8 (IgG2a, κ) monoclonal antibodies and their matching isotypes (Becton Dickinson) to evaluate non specific binding. Briefly, cells (5×10^5) were incubated with CMXRos (50 nM) for the last 30 min in culture medium and then stained with isotypes or specific antibodies (500 ng/ml) in the presence of excess FcR block (Miltenyi) for 15 min before analysis.

2.4. Protein extraction

Cells (30×10^6) were washed twice with cold PBS by centrifugation at $750 \times g$ for 10 min at 4 °C and lysed in 150 μl of RIPA buffer (10 mM Tris–HCl, 140 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 2 mM PMSF, 10 μg/ml pepstatin, 10 μg/ml aprotinin, 10 μg/ml leupeptin, pH 7.5). After 1 h on ice, the lysate was centrifuged at $15{,}000 \times g$ for 15 min at 4 °C and the supernatant was collected. Protein concentration was determined by Bradford assay.

2.5. Isolation of cytosol and nuclear fractions

Thymocytes were washed with PBS and resuspended in 500 μl ice-cold buffer A (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 2 mM DTT, 0.5 mM PMSF, 5 μg/ml pepstatin, 5 μg/ml aprotinin, 5 μg/ml leupeptin, pH 7.8) by gentle pipetting. The cells were allowed to swell on ice for 15 min. 25 μl of 10% NP-40 in buffer A was added and the tubes were vortexed for 20 s. The homogenate was centrifuged for 2 min at $1400 \times g$ at 4 °C. The resulting cytosolic supernatant was removed and the nuclear pellet was resuspended in 50 μl ice-cold buffer C (20 mM HEPES, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 25% glycerol, 2 mM DTT, 0.5 mM PMSF, 5 μg/ml leupeptin, pH 7.9) for 30 min on ice. The nuclear extract was centrifuged for 5 min at $14{,}000 \times g$ at 4 °C and protein content of cytosol and nuclear fractions was determined by Bradford assay.

2.6. Western blotting

Equivalent amounts of protein were electrophoresed on SDS-polyacrylamide gels and electroblotted onto nitrocellulose membrane (Amersham Biosciences). Membranes were blocked and probed with appropriate primary antibodies: GR (M-20, sc-1004), lamin B (sc-6217) (Santa Cruz Biotechnology) and β-actin (A-5441, Sigma). Primary antibodies were detected by appropriate horseradish peroxidase-conjugated secondary antibody and visualized using the enhanced chemiluminescence detection system (Amersham Biosciences).

2.7. Immunofluorescence experiments

Cells (5×10^5) were centrifuged ($200 \times g$ for 10 min) on cytospin slides, fixed with methanol for 20 min at −20 °C and permeabilized with 0.1% Triton X-100 for 10 min and washed again with