Steroid-Independent Translocation of the Glucocorticoid Receptor by the Antidepressant Desipramine

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SUMMARY
The glucocorticoid receptor (GR) is a ligand-regulated transcription factor that in its unactivated form resides primarily in the cytoplasm. After being bound by steroid, the GR undergoes a conformational change and translocates to the nucleus, where it influences gene transcription. Because the GR mediates negative feedback exerted by circulating glucocorticoid hormones on the hypothalamic-pituitary-adrenal (HPA) axis, it has been hypothesized that abnormalities in GR expression and/or function may underlie the HPA axis hyperactivity described in patients with major depression. In further support of this hypothesis, animal studies have shown that long-term in vivo treatment with antidepressants enhances glucocorticoid feedback inhibition, possibly through a direct effect on the GR. To examine this latter possibility, we evaluated translocation of the GR from the cytoplasm to the nucleus after 24-hr in vitro treatment of L929 cells (mouse fibroblasts) with the tricyclic antidepressant desipramine (0.1–10 μM) in the presence or absence of the synthetic steroid dexamethasone. In addition, GR-mediated gene transcription was measured with the use of L929 cells stably transfected with the mouse mammary tumor virus-chloramphenicol acetyltransferase reporter gene. Desipramine was found to (i) induce GR translocation from the cytoplasm to the nucleus in the absence of steroids (with no effect alone on GR-mediated gene transcription) and (ii) potentiate dexamethasone-induced GR translocation and dexamethasone-induced GR-mediated gene transcription. Treatment with desipramine for 24–96 hr had no effect on the expression of GR protein as measured by cytosolic radioligand receptor binding. We suggest that one important aspect of the effects of antidepressants in vivo may be to facilitate GR-mediated feedback inhibition on the HPA axis, by facilitating GR translocation and function, and thereby reverse glucocorticoid hypersecretion in depression.

Hyperactivity of the HPA axis in patients with major depression is one of the most consistent findings in biological psychiatry. Specifically, patients with major depression have been found to exhibit an increased concentration of cortisol in plasma, urine, and cerebrospinal fluid; an exaggerated corticosteroid response to ACTH; and an enlargement of both the pituitary and adrenal glands (1, 2). These HPA-axis alterations are believed to be secondary to hypersecretion of CRF. A hypothesis supported by demonstration of increased concentrations of CRF in the cerebrospinal fluid of depressed patients (i) decrease-regulation of receptors for CRF in the frontal cortex of victims of suicide (presumably secondary to hypersecretion of CRF), and a blunted ACTH response to a CRF challenge (probably reflecting down-regulation of pituitary CRF receptors) (1, 2).

One mechanism by which CRF hypersecretion may occur in major depression is through altered feedback inhibition of CRF release by endogenous glucocorticoids. Data supporting the notion that glucocorticoid-mediated feedback inhibition is impaired in major depression come from a multitude of studies demonstrating nonsuppression of cortisol secretion after administration of the synthetic glucocorticoid Dex (3) and from more recent studies showing a lack of inhibition of ACTH responses to CRF after Dex pretreatment (2).

Because glucocorticoid hormones exert their negative feedback through binding to GR in HPA-axis tissues and the hippocampus (which has been shown to mediate an inhibitory influence on CRF in the paraventricular nucleus) (4), the possibility that depression is associated with a primary alteration in GR number and/or function has been an important consideration regarding the pathophysiology of the de-
pressive disorders. A number of studies have directly examined GR number and function in depression, and although some researchers have observed decreased GR number in depressed patients, the majority have not (5). Nevertheless, studies investigating GR function have demonstrated consistently that peripheral blood mononuclear cells from depressed patients are significantly less sensitive to the inhibitory effects of glucocorticoids on functional end points compared with cells from healthy control subjects (6–8). This in vitro glucocorticoid resistance is consistent with the in vivo data showing nonsuppression of HPA-axis function after Dex.

In further support of the hypothesis that abnormalities in the GR contribute to the pathophysiolo...g of major depression, recent studies have suggested that a possible mechanism by which antidepressants exert their effect is through direct modulation of the GR. In fact, a number of animal studies have shown that long term in vivo treatment with a range of tricyclic and nontricyclic antidepressants or electroconvulsive therapy is capable of enhancing glucocorticoid feedback inhibition (as demonstrated by decreased basal and/or stress-induced glucocorticoid secretion) and/or increasing GR binding and mRNA in key brain regions, including the hippocampus (9–16).Antidepressants have also been found to facilitate glucocorticoid-mediated feedback inhibition and increase GR in animal models of HPA-axis dysregulation, including a transgenic mouse model of impaired GR function (17) and a rat model of early developmental stress that leads to hypersecretion of CRF in response to stress in adulthood. Finally, antidepressant medications have been associated with resolution of HPA-axis alterations (2) and in vitro glucocorticoid resistance (18) in patients with major depression.

Interestingly, a recent study by Rossby et al. (16) demonstrated that GR mRNA up-regulation occurred in rats treated chronically with the tricyclic antidepressant and noradrenaline reuptake inhibitor DMI (desmethylimipramine), even after complete neurotoxic lesioning of noradrenaline neurons. Moreover, an in vitro study using a fibroblast cell line that does not secrete catecholamines has shown that acute DMI treatment was capable of enhancing GR-mediated gene transcription (after 24 hr of treatment) as well as inducing up-regulation of GR protein (after 72 hr of treatment) (19). Taken together, the data suggest that a potentially important mode of action of DMI may be to directly modulate the GR.

The purpose of the current study was to further investigate the mechanism by which antidepressants influence GR function by examining the effect of DMI on the translocation of the GR from the cytoplasm to the nucleus. According to the “nucleocytoplasmic traffic” model, the GR in its unactivated form resides primarily in the cytoplasm, and after being bound by steroid, it undergoes a conformational change (“activation”); dissociates from a multimeric complex, including several hsps; and translocates from the cytoplasm to the nucleus, where it binds to GREs and interacts with other transcription factors (20). We evaluated GR translocation in L929 cells (mouse fibroblasts) that had been treated with the tricyclic antidepressant DMI for 24 hr in the presence or absence of the synthetic steroid Dex. GR translocation was investigated using GR immunostaining, Western immunoblotting of immunoprecipitated GR protein in cytosolic and nuclear fractions, and cytosolic radioligand receptor binding. In addition, GR-mediated gene transcription was measured with the use of L929 cells stably transfected with the MMTV-CAT reporter gene. Our results show that DMI induces the GR to translocate from the cytoplasm to the nucleus in the absence of any endogenous or exogenous steroid and potentiates Dex-induced GR translocation and Dex-induced GR-mediated gene transcription. We observed no effect of 24–96 hr treatment with DMI on the expression of GR protein.

Experimental Procedures

Materials. Unlabeled Dex and DMI were obtained from Sigma Chemical (St. Louis, MO). [6,7,8H]Dex (43.2 Ci/mmol) was obtained from New England Nuclear Research Products (Boston, MA). Human fibronectin was obtained from Becton Dickinson Labware (Franklin Lakes, NJ). The LMCAT cell line was generously provided by Dr. E. R. Sanchez (Department of Pharmacology, Medical College of Ohio, Toledo, OH). RU28362, the GR agonist, was a gift from Roussel-Uclaf (Romainville, France).

Cell culture conditions and drug treatments. Mouse fibroblast cells (L929) and the stably transfected CAT reporter cell line LMCAT [derived from L929 (21)] were maintained in 175-cm² flasks (Becton Dickinson Labware) at 37°C with a 5% CO2/95% air atmosphere. The culture medium for L929 cells was DMEM with 10% (v/v) heat-inactivated fetal bovine serum, and 50 µg/ml streptomycin. For immunostaining, cells were grown in DMEM with charcoal-extracted (1% activated charcoal 0.1% dextran) calf bovine serum. For LMCAT cells, culture medium was DMEM with 10% (v/v) charcoal-extracted, heat-inactivated (56°C, 30 min) newborn calf serum and 0.2 mg/ml G418 (Geneticin) antibiotic.

For the CAT assay, LMCAT cells were cultured in fibronectin-coated six-well plates and grown for 12 hr (final confluence, 95%) before drug treatment. For binding assays and immunoprecipitation/Western blotting, L929 cells were cultured in fibronectin-coated 175-cm² flasks for 48–72 hr (final confluence, 95%) before drug treatment. For immunostaining, cells were transferred into fibronectin-coated chamber slides (Nunc, Naperville, IL) for 12 hr to obtain a final confluence of ~70% and then drug treated.

Treatment of both L929 cells and LMCAT for all assays consisted of incubation with fresh medium containing final concentrations of DMI (0.1–10 µM) and Dex (10 nM to 10 µM). This range of DMI concentrations includes the therapeutic plasma levels of DMI (22) as well as concentrations that have been used in other studies that investigated the in vitro effects of this drug (23).

Immunostaining procedures and fluorescence quantitation. Cells were fixed/permeabilized with methanol at ~20°C for 10 min, followed by a 30-min incubation with 5% BSA to block nonspecific antibody binding. Cells were then incubated with the rabbit polyclonal antibody 57 (GR57) against the human GR (Affinity Bioreagents, Golden, CO) at a concentration of 5 µg/ml in 2% NDS in PBS for 30 min at room temperature followed by overnight incubation at 4°C. The next day, cells were incubated with biotin-conjugated donkey anti-rabbit antibody (Jackson Immunoresearch Laboratories, West Grove, PA) at a concentration of 10 µg/ml in NDS for 1 hr, followed by incubation with the FITC-conjugated streptavidin (Jackson Immunoresearch Laboratories) at a concentration of 9 µg/ml in PBS for 1 hr (in darkness). Incubations and washes were performed at room temperature except where otherwise specified. Two washes with PBS buffer were performed between all steps. Cells were mounted with a glass coverslip using the Slowfade-Light Antifade reagent in glycerol buffer (Molecular Probes, Eugene, OR). Microscopic examination was performed using a Nikon Microphot-SA microscope with a Nikon PlanApo 200.75 objective. The UV source was a Nikon Mercury Lamp HB-10101 AP.
The protocol for the quantitative analysis of fluorescence in the cytoplasm and nucleus was developed in consultation with Dr. David E. Wolf (Cell Biology Group, Worcester Foundation for Biomedical Research, Worcester, MA). Microscopic fields were captured under both light and fluorescence illuminations and transformed into digital images to be shown on a computer screen. Sampling was performed on two to four different areas of each well. The microscope and camera settings were maintained constant between all the experimental conditions, and no adjustment of the gray scale was performed in the images. An ROI was selected in the cytoplasm and in the nucleus of each cell of the section. The intensity value of each pixel within the ROI ranged between 0 and 255 and was proportional to the number of fluorescent photons emitted from the corresponding point in the specimen. After subtraction of the background (no cells), the mean intensity value of the ROI was calculated. This value represented a measure of the fluorescence detected from each ROI and could be used to make comparisons between the same compartments under different conditions. Several steps were included to have an objective and accurate series of measurements. First, the ROI was initially outlined “blind” to the fluorescent signal (and to the treatment condition) using light microscopy images; afterward, the selections were superimposed onto the corresponding fluorescent image, and the fluorescence intensity in the region was quantified. Second, the ROI was defined using the oval tool of the image software, and its width was kept constant. Third, the entire cell and nucleus were demarcated manually, and the resulting areas (number of pixels) were calculated to control for possible changes in the shape of the cells.

Preparation of L929 cells for immunoprecipitation/Western blotting and binding assay. After the incubation with drugs in 175-cm² flasks, cells were washed and scraped into cold HBSS, transferred to 50-ml tubes, pelleted at 700 × g for 10 min, resuspended in HBSS for three to five consecutive washes, and then homogenized as described below.

Cellular fractionation and immunoprecipitation of GR protein. Cellular fractionation and immunoprecipitation of GR were performed according to a procedure that generates soluble cytosolic and nuclear fractions (modified from Ref. 21). Cells were harvested as indicated above and counted using a Coulter Counter model ZF (Coulter Electronics, Hialeah, FL). Equivalent numbers of cells were resuspended in hypotonic buffer (consisting of 10 mM HEPES, 5 mM EDTA, 20 mM sodium molybdate, pH 7.4, with a cocktail of protease inhibitors, including 0.01 mg/ml aprotinin, 0.01 mg/ml leupeptin, 0.01 mg/ml soybean trypsin inhibitor, and 0.16 mg/ml phenylmethylsulfonyl fluoride) followed by Dounce homogenization. After centrifugation at 1000 × g for 5 min, the cytosolic (supernatant) and nuclear fractions (pellet) were saved. The cytosolic fraction was centrifuged again at 20,000 × g for 10 min, and the resulting supernatant was collected for salt extraction. The nuclear fraction was resuspended in hypotonic buffer and centrifuged at 900 × g for 5 min, and this pellet was resuspended again in hypotonic buffer and collected for salt extraction. Each fraction was then solubilized using 1 hr of salt extraction (5 mM EDTA, 20 mM sodium molybdate, 1 M NaCl). The nuclear fraction was centrifuged again (20,000 × g, 10 min), and the supernatant was collected. Finally, the rabbit polyclonal antibody GR59 (GR59) against the human GR (Affinity Bioreagents) was added to the samples at a final concentration of 2 µg/ml for an overnight incubation. All of these procedures were carried out at 4°C. The GR/antibody immunocomplexes were isolated by the addition of protein A/Sepharose, followed by two centrifugation/wash steps, and extraction in sodium dodecyl sulfate sample buffer by heating at 95°C.

Gel electrophoresis and quantitative Western blotting. Samples were resolved by electrophoresis in 4–7% sodium dodecyl sulfate-polyacrylamide gel. After transfer of the protein content to an Immobilon-P membrane (Millipore, Bedford, MA), the relative amount of GR protein in the cytosolic and nuclear fractions was determined using Western blot analysis (modified from Ref. 21). To ensure completeness of transfer, all gels were stained with Coomassie blue, and Immobilon P membranes were stained periodically with India ink to ensure uniformity of transfer and loading. The membrane was incubated with 0.2% BSA in TBS with 0.1% (v/v) Tween-20 for 5 min (blocking step) followed by incubation overnight with the rabbit polyclonal antibody 57 (GR57) against the human GR (0.5 µg/ml; Affinity Bioreagents) in 0.2% BSA in TBS with 0.1% (v/v) Tween-20. The GR57 antibody recognizes a different epitope on the GR (amino acids 346–367) than the GR59 antibody used for immunoprecipitation (amino acids 245–259). After the application of purified horseradish peroxidase-labeled goat anti-rabbit IgG second antibody (1:25,000 in 10% (v/v) fetal bovine serum in TBS with 0.1% (v/v) Tween-20; Kirkegaard and Perry Laboratories, Gaithersburg, MD), the GR protein was visualized using the Enhanced Chemiluminescence detection system (Amersham, Buckinghamshire, UK).

Gel images of the blots were scanned and then analyzed using a specific NIH Image software algorithm that subtracts the background signal and integrates the width and absorbance of each band as an area-under-the-curve graphic. The corresponding value (dimension of the area-under-the-curve) allowed a relative quantification of the amount of GR protein in the cytosolic and nuclear fractions under the different conditions.

GR binding assay. GR binding was determined using a previously described in vitro cytosolic exchange assay (24). Cells were fractionated using a freeze/thaw procedure in a volume of 0.7 ml of binding buffer (10 mM Tris, 1 mM EDTA, 20 mM molybdic acid, 5 mM dithiothreitol, and 10% glycerol in double-distilled water, pH 7.4 at 4°C), yielding an approximate final protein concentration of 0.5–1.5 mg/ml of cytosol.

After centrifugation at 105,000 × g for 60 min at 4°C, the supernatant-cytosol was added to incubation solutions containing radiolabeled 3H-Dex with or without unlabeled competitor. Bound radiolabeled steroid was separated from unbound steroid by filtration through minicolumns containing 1.25 ml of LH-20 Sephadex (Pharmacia, Piscataway, NJ). Scintillation fluor (Ultima Gold, Packard, Meriden, CT) was added to elute containing the bound fraction of steroid, and tritium 3H-radioactivity was determined in a Wallac LKB 1209 liquid scintillation counter (Upssala, Sweden). For single-point assays, GR receptor binding was defined as the amount of total 3H-Dex (10 nM) binding displaced by the selective GR agonist RU28362 (0.5 µM). Several steps were included to reduce the amount of residual drug present in the incubation mixture; these included three to five HBSS washes after drug treatment and an additional 50-fold dilution of cytosol before incubation with the radioligand. Incubations with 3H-Dex were carried out at 4°C for 18–22 hr to allow full exchange of the radiolabeled Dex with any residual competitor.

Specific binding was expressed as fmol/mg of cytosolic protein. Protein content for all samples was determined according to the method of Bradford with use of BSA as a standard as described previously (24).

Digital processing system. A digital processing system that included a computer station, image analysis software, and two devices for image acquisition (a camera and scanner) was used to acquire, store, and process the microscopic and Western blot images, as well as perform the quantitative analyses.
Fig. 1. GR translocation after treatment with DMI and Dex, alone or in combination, using immunostaining of GR and quantification of fluorescence in the cytoplasm and in the nucleus. L929 cells were grown in steroid-free medium and treated with vehicle (A), DMI (10 \( \mu \)M) (B) for 24 hr, Dex (10 nM) (C) for 1.5 hr, and DMI (10 \( \mu \)M) (D) for 24 hr, followed by coincubation of DMI (10 \( \mu \)M) plus Dex (10 nM) for 1.5 hr. GRs were immunostained using the anti-GR polyclonal antibody GR57. Quantitative analysis of fluorescence (FITC) was performed in blindly selected regions from the cytoplasm and nucleus using digital image analysis. Results are based on quantification of \( \sim \)800 cells from three to five.
**Results**

**Effect of DMI on GR immunostaining.** To examine the effect of DMI on GR nucleocytoplasmic traffic, we used a fluorescence/immunostaining procedure with an anti-GR polyclonal antibody coupled with quantitative analysis of fluorescence in the cytoplasm and nucleus of cells and digital image analysis (see Experimental Procedures). Cells were grown in steroid-free conditions (charcoal-extracted serum) and treated with (a) vehicle, (b) DMI (10 μM) for 24 hr, (c) Dex (10 nM) for 1.5 hr, and (d) DMI (10 μM) for 24 hr followed by coincubation of DMI (10 μM) plus Dex (10 nM) for 1.5 hr. We have shown this low dose of Dex (10 nM) to induce only partial translocation of the GR in this assay, therefore providing a useful tool for testing the putative effects of DMI on Dex-induced GR translocation.

L929 cells stained for the GR after the various treatments are presented in Fig. 1, together with quantitative analysis from ~800 cells. We evaluated the fluorescence intensity in the cytoplasm and nucleus (expressed as percentage of the fluorescence intensity of vehicle-treated cells).

After treatment with vehicle (A), the pattern of staining was heterogeneous among cells, with a diffuse staining in both the cytoplasm and nucleus. In the majority of cells, the fluorescent signal was more intense in the cytoplasm than in the nucleus, although a few cells with brighter nuclei were present. Nucleoli were never stained. After treatment with DMI (10 μM) (B), Dex (10 nM) (C), or DMI (10 μM) plus Dex (10 nM) (D), cells presented more intense nuclear staining, suggesting that all treatments induced translocation of the GR from the cytoplasm to the nucleus. Cells treated with DMI (10 μM) plus Dex (10 nM) showed a greater effect (brighter nuclei) compared with those treated with either DMI or Dex alone. Quantitative analysis showed that alone, DMI (10 μM) or Dex (10 nM) induced a decrease of the GR fluorescent signal in the cytoplasm of ~20% and ~26%, respectively, whereas coincubation of DMI (10 μM) plus the same dose of Dex (10 nM) caused a ~39% decrease in the cytoplasmic fluorescence. In the nucleus, DMI (10 μM) or Dex (10 nM) alone induced an increase in the signal of ~25% and ~41%, respectively, whereas coincubation of DMI (10 μM) plus Dex (10 nM) led to a ~67% increase in nuclear fluorescence.

To control for the specificity of the immunostaining for GR, a series of experiments were conducted in which the primary antibody anti-GR (GR57) was either preabsorbed against the immunogen peptide (PEP-001; Affinity Bioreagents) or substituted by NDS, or the second antibody (anti-rabbit IgG) was substituted with NDS; controls were always negative. Fixed but unstained cells showed only minimal autofluorescence. There were no relevant differences among the various treatment conditions in the morphology of the cells or in the area of the entire cell or nucleus.

To further characterize the effects of DMI on GR translocation, a dose-response experiment was conducted in which cells were treated for 24 hr with vehicle or DMI (0.1–10 μM). Moreover, to investigate whether the GR translocated by DMI treatment was capable of recirculating back to the cytoplasm after drug withdrawal, cells were also treated with DMI (10 μM) for 24 hr and then allowed to incubate in medium without DMI for 24 hr. The results of quantitative fluorescence in ~900 cells are presented in Fig. 2. There was only a minimal effect of DMI (0.1 μM) on GR translocation, whereas a dose-dependent effect was present after treatment with DMI (1 μM) and DMI (10 μM). GR translocation was reversed in cells that recovered for 24 hr after DMI treatment.

**Effect of DMI on GR using immunoprecipitation/Western blot.** We evaluated GR nucleocytoplasmic traffic in cytoplasmic and nuclear fractions obtained by cell rupture, followed by immunoprecipitation/Western blot of the receptor and densitometric analysis of the GR band.

Cells were treated with (a) vehicle, (b) DMI (10 μM) for 24 hr, (c) Dex (100 nM) for 1.5 hr, and (d) DMI (10 μM) for 24 hr followed by DMI (10 μM) plus Dex (10 nM) for 1.5 hr. In this assay, Dex (10 nM) was found to induce only minimal translocation of the GR (~15% decrease in the cytoplasm and no increase in the nucleus; data not shown), thus suggesting that this technique is less sensitive to a small amount of GR translocation than the previously described immunostaining. Therefore, we investigated the effect of DMI on Dex-induced GR translocation using a higher dose of Dex (100 nM), which induced partial but clear GR translocation.

Representative Western blots are presented in Fig. 3 with results of the densitometric quantification of the GR bands from five independent experiments (expressed as percentage of the signal in the vehicle-treated cells). The rabbit polyclonal antibody 57 (GR57) against the human GR (Affinity Bioreagents) recognized a prominent band at ~97 kDa, which is consistent with similar experiments using a mono-

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CAT reporter cell line and CAT assay. The LMCAT cell line (derived from L929 cells) is stably transfected with the MMTV-CAT reporter plasmid. Expression of CAT activity by these cells is under hormonal control by virtue of several GREs residing within the MMTV promoter which lies upstream of the CAT reporter gene (21).

Measurement of CAT enzyme activity was performed using a liquid scintillation counting detection system (Promega, Madison, WI) according to the manufacturer’s instructions. Briefly, cell extracts were obtained using a Tris buffer (0.25 M Tris-HCl, pH 8.0) freeze/thaw procedure, followed by 60° heating for 10 min to inactivate endogenous deacetylase activity. After centrifugation (20,000 × g for 2 min), supernatants were transferred to fresh tubes and processed for CAT enzyme activity. Each reaction was initiated by adding the cofactor n-butylry Coenzyme A to tubes containing cell extracts and radiolabeled [3H]-chloramphenicol. The CAT reaction was stopped, and the butyrylated forms of [3H]-chloramphenicol were separated by three consecutive extractions with mixed xylene. The extracts were transferred to vials for liquid scintillation counting. The cpm measured in each sample represents the butyrylated fraction of the enzyme (as determined by a standard curve) and is directly proportional to the CAT gene expression.

Protein content for all samples was determined according to the method of Bradford with use of BSA as a standard (24).

**Statistical analysis.** Data are presented as mean ± standard error and were analyzed using one-way and two-way analyses of variance. When the analysis of variance revealed a significant main effect of treatment condition, both a conservative (Student-Newman-Keuls) and a powerful (least significant difference) post hoc test were used for between-group comparisons.
clonal antibody to the GR (21). In cells treated only with vehicle (Fig. 3, A and B, lanes 1 and 2), the majority of GR was found in the cytosolic fraction. Some GR shifted from the cytosolic to the nuclear fraction in cells treated with DMI (10 \( \mu M \)) (Fig. 3A, lanes 3 or 4) or Dex (100 nM) (Fig. 3B, lanes 3 and 4). Compared with Dex alone, a larger shift of GR was evident after treatment with DMI (10 \( \mu M \)) plus the same dose of Dex (100 nM) (Fig. 3B, lanes 5 and 6). Densitometric quantification showed that DMI (10 \( \mu M \)) and Dex (100 nM) induced a decrease of the GR in the cytoplasm of \( 
abla 25\% \) and \( 
abla 27\% \), respectively, whereas coincubation of DMI (10 \( \mu M \)) plus Dex (100 nM) caused \( 
abla 45\% \) decrease. In the nucleus, DMI (10 \( \mu M \)) and Dex (100 nM) induced an increase in the signal of \( 
abla 33\% \) and \( 
abla 116\% \), respectively, whereas coincubation of DMI (10 \( \mu M \)) plus Dex (100 nM) led to \( 
abla 150\% \) increase in nuclear GR.

Treatment with a high dose of Dex (10 \( \mu M \)) resulted in GR mostly localized in the nuclear fraction (Fig. 3B, lanes 7 and 8).

It should be noted that there is clearly more GR protein in the cytosolic than the nuclear compartment under sham (vehicle) conditions (Fig. 3, A and B, lanes 1 and 2). Nevertheless, each compartment (cytoplasm and nucleus) in the vehicle condition was arbitrarily assigned a value of 100% to allow quantitative comparisons among the various treatment groups within a given compartment. In addition, this approach was taken to allow comparison of the results of the Western blot studies with the immunostaining experiments in which cytosolic and nuclear fractions in the sham (vehicle) condition were also each set at 100% for within-group comparisons. The immunostaining experiments required normalization of the data in each compartment (as opposed to normalization of all data to the cytosolic compartment, in which the majority of the receptor is located under sham conditions) because assessments of total GR within a given compartment cannot be made accurately with this technique (i.e., assessments included average fluorescence intensity over a standardized area in the cytoplasmic and nuclear compartment; significant differences were not detected).

Fig. 2. Dose-related effect of DMI on GR translocation and recirculation of the translocated GR to the cytoplasm after DMI withdrawal using immunostaining of GR and quantification of fluorescence in the cytoplasm and the nucleus. L929 cells were grown in steroid-free medium and treated for 24 hr with vehicle, DMI (0.1, 1, or 10 \( \mu M \)), or with DMI (10 \( \mu M \)) for 24 hr and then allowed to incubate in medium without DMI for 24 hr. GRs were immunostained using the anti-GR polyclonal antibody GR57. Quantitative analysis of fluorescence (FITC) was performed in blindly selected regions from the cytoplasm and nucleus using digital image analysis. Quantitative fluorescence in \( \nabla 900 \) cells from three independent experiments is presented, and results are expressed as mean \( \pm \) standard error percentage of the signal in vehicle-treated cells. *, Significant (\( p < 0.05 \)) difference versus vehicle using Student-Newman-Keuls post hoc analysis.

Fig. 3. GR translocation after treatment with DMI and Dex alone or in combination using Western blot of the GR in the cytoplasmic and nuclear compartments and densitometric quantification of GR bands. A, L929 cells were treated with vehicle (lanes 1 and 2) or DMI (10 \( \mu M \)) for 24 hr (lanes 3 and 4). B, L929 cells were treated with vehicle (lanes 1 and 2), Dex (100 nM) for 1.5 hr (lanes 3 and 4), and DMI (10 \( \mu M \)) for 24 hr, followed by coincubation of DMI (10 \( \mu M \)) plus Dex (100 nM) for 1.5 hr (lanes 5 and 6). Cells treated with a high dose of Dex (10 \( \mu M \)) are presented to demonstrate maximal translocation of the GR in this assay system (lanes 7 and 8). Cells were fractionated using a cell-rupturing procedure that generates soluble cytosolic (C) and nuclear (N) fractions. GR present in the fractions was purified with immunoprecipitation using the anti-GR polyclonal antibody GR59 and analyzed by quantitative Western blot using the anti-GR polyclonal antibody GR57 (directed toward a different epitope than the GR59) and a horseradish peroxidase-conjugated counterantibody. The GR57 recognized a prominent band at \( 
abla 97 \) kDa. C, quantification of GR bands was based on densitometric analysis from five independent experiments, and results are expressed as mean \( \pm \) standard error percentage of the signal in vehicle-treated cells. M, molecular mass marker. *, Significant (\( p < 0.05 \)) difference versus vehicle using Student-Newman-Keuls post hoc analysis.
see Experimental Procedures). Therefore, for consistency of data presentation, both Western blot and immunostaining experiments are presented as comparisons within each compartment with data normalized to 100% in the respective compartment in the sham condition.

**Effect of DMI on cytosolic GR binding.** We evaluated 3H-Dex binding sites using an in vitro exchange assay of cytoplasmic homogenates. Activation and translocation of the GR from the cytoplasm to the nucleus are associated with decreased GR binding in the cytosolic fraction; therefore, cytosolic GR binding can be used as a further method for the evaluation of receptor nucleocytoplasmic traffic. However, when most of the receptor is in the unactivated state, cytosolic GR binding is also a measure of the amount of receptor in the cells, and therefore its evaluation may be useful to investigate expression of GR protein.

To further investigate the effect of DMI on GR nucleocytoplasmic traffic, L929 cells were treated with (a) vehicle, (b) DMI (10 μM) for 24 hr, (c) Dex (10 nM) for 1.5 hr, and (d) DMI (10 μM) for 24 hr followed by DMI (10 μM) plus Dex (10 nM) for 1.5 hr. Dex (10 nM) was chosen for this experiment because we have shown this dose to induce only partial translocation of the GR in this assay; furthermore, by starting with this low dose of Dex, we avoided any appreciable carryover of drug from the cell treatment step into the exchange assay.

As shown in Fig. 4, all treatments induced a decrease in cytosolic GR binding. Consistent with data from the fluorescence/immunostaining and immunoprecipitation/Western blot experiments, DMI (10 μM) and Dex (10 nM) induced a decrease of the cytosolic GR binding of ~17% and ~15%, respectively, whereas coincubation of DMI plus the same dose of Dex (10 nM) caused a greater decrease in cytoplasmic GR binding (~30%).

Because in a previous study in a fibroblast cell line, increased cytosolic GR binding (up-regulation) had been reported in cells treated with DMI for 72–96 hr (19), we investigated the effects of longer DMI treatment on cytosolic GR binding. L929 cells were treated with (a) vehicle and (b) DMI (10 μM) for 48–96 hr. In the initial experiments, cytosolic extracts for binding analysis were prepared immediately after the DMI treatment. In a second set of experiments, the cells were allowed to incubate for 24 hr after the DMI treatment in medium without DMI before preparation of cytosolic extracts. The latter experiment was conducted to control for the effects of DMI on GR nucleocytoplasmic traffic because if a receptor was constantly being driven into the nucleus, GR up-regulation may be masked. Of note is that our data had demonstrated previously that 24 hr of recovery allowed re-circulation of GR from nucleus to cytoplasm after DMI treatment (see Fig. 2). Results are presented in Table 1. GR cytosolic binding was consistently ~10–20% lower in cells treated with DMI than in the untreated cells. Moreover, GR binding in cells examined after DMI treatment plus 24-hr recovery was similar or lower than GR binding in untreated cells.

In a third experiment, we evaluated whether DMI was exerting its effect on GR translocation by influencing the binding of 3H-Dex to the GR. Cytosolic extracts of untreated L cells were examined in an exchange assay with 3H-Dex (10 nM) in the presence or absence of a 1000-fold excess of DMI (10 μM). No differences were found in GR binding in incubates containing 10 μM DMI (1063 ± 72.1 fmol/mg of protein) compared with incubates that did not (931 ± 23.1 fmol/mg of protein) (p = 0.33), suggesting that changes in GR binding in DMI-treated cells were not due to a direct effect of DMI on the capability of the GR to bind 3H-Dex.

Protein concentrations were examined in every sample, and no effect of DMI treatment on protein content was detected.

**DMI effects on GR-mediated gene transcription.** To determine the functional correlates of DMI-induced changes in nucleocytoplasmic traffic, we examined GR-mediated gene transcription in L929 cells stably transfected with a CAT enzyme reporter gene that is under hormonal control by virtue of several GREs residing in the upstream MMTV promoter (LMCAT cells).

According to our data on GR nucleocytoplasmic traffic, DMI alone induced GR translocation in the absence of steroids, and cells treated with DMI plus Dex consistently

![Graph](Image)

**Fig. 4.** Cytosolic GR binding after treatment with DMI and Dex, alone or in combination. L929 cells were treated with vehicle, DMI (10 μM) for 24 hr, Dex (10 nM) for 1.5 hr, and DMI (10 μM) for 24 hr, followed by coincubation of DMI (10 μM) plus Dex (10 nM) for 1.5 hr. Cell lysates were centrifuged, and the supernatant-cytosol was added to incubation solutions containing radiolabeled 3H-Dex for the exchange assay. Values are presented as mean ± standard error percentage of GR binding in vehicle-treated cells from five independent experiments. *, Significant (p < 0.05) difference versus vehicle using Student-Newman-Keuls post hoc analysis. +, Significant (p < 0.05) difference versus Dex (10 nM) using the least significant difference test.
showed greater GR translocation compared with cells treated with the same dose of Dex alone. Therefore, we tested the effect of the same treatment protocol on GR-mediated gene transcription in the absence or presence of various doses of Dex. LMCAT cells were grown in steroid-free medium and treated with (a) vehicle, (b) DMI (10 μM) for 24 hr, (c) Dex (10 nM to 10 μM) for 1.5 hr, and (d) DMI (10 μM) for 24 hr followed by coincubation of DMI (10 μM) plus Dex (10 nM to 10 μM) for 1.5 hr.

Results are presented in Fig. 5 and expressed as relative CAT activity (percentage of maximal effect). We found that DMI alone did not influence GR-mediated gene transcription. Moreover, for every concentration of D, pretreatment with DMI caused a decrease in Dex-induced GR-mediated gene transcription compared with cells treated with the same dose of Dex alone. Inhibition of Dex-induced GR-mediated gene transcription ranged from ~25% (10 nM Dex) to ~50% (1–10 μM Dex). We confirmed these findings by performing different sets of slightly modified experiments, and we consistently found the same results. For example, we found no induction of CAT activity after treatment with DMI alone in cells incubated with DMI (1 μM) instead of DMI (10 μM) or treated with DMI (1–10 μM) for 48 hr instead of 24 hr. Moreover, these results were not due to carryover of DMI from the treatment step into the lysates processed for CAT enzyme activity because similar findings were obtained if cells were washed thoroughly after the 24-hr DMI treatment and then incubated for 1.5 hr in fresh medium without DMI (in the presence or absence of Dex). Maximal GR-mediated gene transcription in this assay (after 1.5 hr with 10 μM Dex) was ~20-fold compared with vehicle-treated cells. Interestingly, our data showing inhibition of Dex-induced GR-mediated gene transcription in cells pretreated with DMI in steroid-free conditions were not consistent with results of a previous study by Pepin et al. (19) showing that pretreatment with DMI in cells grown with non-charcoal-extracted serum induced potentiation of Dex-induced GR-mediated gene transcription. Because of this apparent contradiction, we speculated that in vivo (animals or humans) or in vitro with steroid-containing medium, DMI would exert its effects in the presence of glucocorticoid hormones, and therefore it is possible that the steroid-free conditions in our in vitro experiments did not allow DMI to elicit its full effect on GR function. To further address this issue, we coincubated cells simultaneously with DMI (1–10 μM) plus Dex (10 nM to 10 μM) for 24 hr (Fig. 6). For all concentrations of D, simultaneous cotreatment with DMI induced a dose-dependent increase in GR-mediated gene transcription. This effect was particularly intense in cells treated with 10 nM Dex (~80% of potentiation) and was still present in cells treated with 10 μM Dex (~25% of potentiation). Maximal GR-mediated gene transcription induced by 24-hr treatment with 10 μM Dex in this assay was ~180-fold induction compared with vehicle-treated cells. Protein concentration was examined in every LMCAT sample, and no effect of DMI treatment on protein content was detected.

Discussion

DMI facilitates GR function without affecting expression of GR protein. We conducted the current study to investigate the effects of the tricyclic antidepressant DMI on GR translocation and function. We chose to use an in vitro experimental system with the mouse fibroblast cell line L929 to investigate effects of DMI that were presumably independent from the blocking of norepinephrine reuptake (the mechanism by which this and a number of other antidepressants are believed to exert their therapeutic action). We used a panel of quantitative assays to investigate GR nucleocytoplasmic traffic, GR expression, and GR-mediated gene transcription. Our results demonstrate that 24-hr treatment with DMI induced translocation of the GR from the cytoplasm to the nucleus in the absence of steroid hormones and potentiated Dex-induced GR translocation. Moreover, 24 hr of simultaneous coincubation of cells with DMI plus Dex led to a substantial potentiation of GR-mediated gene transcription compared with cells treated with the same doses of D alone. Finally, evaluation of cytosolic GR binding in cells treated with DMI for 24–96 hr provided no evidence of GR up-regulation under these conditions.

Interestingly, long term treatment with antidepressants in animals has been shown to induce up-regulation of GR in various areas of the brain, as demonstrated by increased GR immunoreactivity, increased GR binding, and increased GR mRNA (9–17). Therefore, the increased glucocorticoid-mediated negative feedback on the HPA axis described in animals that have been treated chronically with antidepressants has been interpreted as a consequence of the increased availability of receptors, which is not usually evident until approximately 10 days after antidepressant treatment. However, our data suggest an alternative model in which DMI in vivo may facilitate the translocation and activation of the GR by circulating hormones, thereby increasing negative feedback on the HPA axis before changes in receptor number. Of
course, increased feedback on the HPA axis and up-regulation of the GR after chronic antidepressant treatment in vivo are related findings because increased feedback (due to the facilitated activation of the receptor) would cause lower baseline and peak levels of circulating glucocorticoid hormones, which in turn could lead to up-regulation of the GR. Therefore, the facilitation of GR translocation (and function) may represent the molecular mechanism by which antidepressants normalize HPA-axis abnormalities in depressed patients, and GR up-regulation may be the consequence rather than the cause.

Of note is that some data from animal studies support this alternative model in which antidepressants exert their primary effect through changes in GR function (GR translocation) and not expression. For example, a study by Montkowski et al. (25) examined the effect of antidepressant treatment using a transgenic mouse model of depression with decreased expression of GR, hyperactivity of the HPA axis, and behavioral deficits indicative of cognitive impairment. Interestingly, Montkowski et al. (25) demonstrated that long-term antidepressant treatment with moclobemide (an antidepressant of the monoamine oxidase inhibitor class) induced normalization of the HPA axis and the behavioral deficits in these animals in the absence of any changes in GR binding. Moreover, consistent with our in vitro data, one study showed that GR binding was decreased in the hippocampus of rats treated chronically with the tricyclic antidepressant amitriptyline after 3 and 7 days of treatment before an increase in GR binding, which was seen after 5 weeks of amitriptyline treatment (13). In addition, treatment of rats with the tricyclic antidepressant imipramine has been found to increase GR immunoreactivity in the nucleus of brain cells (9). Taken together, these findings suggest that increased activation and translocation of the receptor from the cytoplasm to the nucleus may be an important mechanism by which antidepressants directly regulate the GR.

In a study by Pepin et al. (19) conducted in mouse fibroblasts (LTK⁺ cell line), DMI was found to stimulate promoter activity of the GR gene (at 24 hr) and increase GR binding at 72 hr. Based on these data, the authors concluded that DMI may act directly to stimulate the transcription of the GR gene and induce GR up-regulation.

We conducted two series of controlled experiments to evaluate the effects of 48–96-hr of DMI treatment on GR binding in another mouse fibroblast cell line (L929 cells), and in both cases we found no evidence of DMI-induced increases in GR binding. In the first experiment, cells were fractionated immediately after DMI treatment. However, on the basis of the GR up-regulation noted previously by Pepin et al. (19), we suspected that the DMI-induced translocation of the GR to the nucleus might mask receptor up-regulation. Therefore, in the second experiment, cells were washed thoroughly after DMI treatment and allowed to recover for 24 hr in fresh medium without DMI. In fact, immunostaining data had demonstrated that DMI-induced translocation of the GR was reversed after 24 hr of recovery in the absence of DMI (see Fig. 2). Moreover, previous studies from different laboratories have described that recirculation of the GR after activation by hormones (26) or heat shock (21) occurs within 24 hr.

We cannot rule out the possibility that specific characteristics of the cell lines and/or experimental conditions may account for these discrepant findings; for example, the LTK⁺ cells exhibited very low levels of GR receptors (25–80 fmol/mg of protein) compared with the L cells in the present report (1000–1500 fmol/mg of protein) and other mouse tissues we have examined previously, including the thymus (~800 fmol/mg of protein), spleen (~500 fmol/mg of protein), and cortex (~400 fmol/mg of protein) (24). In addition, it is noteworthy that in vivo evidence supporting the idea that GR up-regulation can occur after short-term treatment with antidepressants is lacking. For example, two studies in vivo have shown that GR mRNA in the brain of laboratory animals is unchanged from base-line after 48 hr of treatment with amitriptyline (12) or DMI (16), whereas one study, as mentioned previously, found decreased GR binding after 3 and 7 days of treatment with amitriptyline (13). A possible explanation for these discrepancies is that LTK⁺ cells in the report by Pepin et al. (19) seem to be grown and treated in non-charcoal-extracted serum, and therefore it is possible that facilitation of GR activation by endogenous steroids may occur in cells treated with DMI alone. If this were the case, we cannot exclude that the activated GR may have led to positive autoregulation (autoinduction) (i.e., increased GR mRNA and increased GR binding after treatment with recep-
tor agonists). In fact, it has been reported previously that short term in vitro treatment of several cell lines (27) as well as primary neuronal cultures (28) with GR agonists may induce GR mRNA and GR protein and that these effects are mediated by the activated GR (27). Interestingly, in the same study by Pepin et al. (19), the authors found increased DEX-induced GR-mediated gene transcription (using LTK+ cells stably transfected with the MMTV-CAT reporter gene) after treatment with DMI for 24 hr, whereas GR binding was not increased until after 72 hr of DMI treatment.

**Steroid-independent modulation of the GR.** Steroid-independent activation of hormone receptors has been described previously, most notably by O’Malley et al. (29), who characterized a pathway leading to activation of the progesterone receptor by dopamine. The concept of “steroid-independent” regulation indicates that steroid receptor function is regulated not only by the specific features of the ligand (natural or synthetic hormones or antagonists) but also by other biochemical events driven by unrelated compounds and acting on different pathways.

Recent in vitro and in vivo research has demonstrated that GR function can also be influenced by a myriad of nonsteroid compounds, including the immunosuppressants cyclosporine and FK506 (30), the cytokine interleukin-1 (31), and different experimental conditions such as heat and chemical shock (21, 32) or viral infection (24).

Clearly, by using in vitro systems (i.e., charcoal extraction) or certain in vivo experimental conditions (i.e., adrenalectomy), it is possible to elicit effects that are induced by these modulators in the absence of any glucocorticoid (e.g., the DMI-induced GR translocation reported here); however, these are artificial conditions, and the presence of at least a minimal dose of steroid (a situation more similar to “physiological” in vivo conditions) is usually required to obtain full manifestation of the effects of these modulators. For example, heat and chemical shock have been shown to induce GR translocation in L929 cells (32), although alone they do not have an effect on GR-mediated gene transcription while strongly potentiating GR-mediated gene transcription induced by Dex (21). Nevertheless, the data from experiments involving steroid-free environment represent a useful tool for dissecting the mechanisms involved.

Consistent with this model, we demonstrated that in the absence of steroids, DMI is capable of inducing GR translocation but does not activate GR-mediated gene transcription. Moreover, we found that treatment with DMI in the absence of steroid before treatment with Dex leads to inhibition of GR-mediated gene transcription compared with cells treated with Dex alone (in contrast to the potentiation that occurred when cells were treated simultaneously with DMI and Dex). These findings are consistent with other data from our and other laboratories showing that translocation is a distinct step from the functional activation of the GR. For example, the GR antagonists RU486 and RU40555 have been shown to induce full nuclear localization of the GR but have no or minimal agonistic activity (33). Moreover, it is well known that the GR that dissociates from the cytoplasmic multimeric complex with the hsps and translocates to the nucleus becomes incapable of rebinding ligand; this has been demonstrated after both steroid-induced GR translocation (34) and heat shock-induced GR translocation (21, 32). Therefore, it is not surprising that pretreatment of cells with DMI alone would inhibit the effects of the subsequent Dex treatment. Because DMI induces some GR to translocate into the nucleus (which will be incapable of binding Dex), less receptor will be available in the cytoplasm when the cells are treated with Dex, resulting in a decreased response.

**Mechanisms of DMI effect on GR.** We have confirmed and extended findings from the previous report by Pepin et al. (19) showing that DMI influences GR function in cells (mouse fibroblasts) that do not secrete catecholamines, endorsing the fact that these in vitro effects are unrelated to the well characterized ability of DMI to inhibit the norepinephrine transporter.

One possibility is that DMI may have a direct effect on one or more hsps and facilitate the dissociation of the receptor from the hsp complex. In fact, there are intriguing similarities between the effects of DMI and those of heat or chemical shock in L929 cells (21, 32, 35) (i.e., induced translocation of GR in the absence of steroids, no effect on GR-mediated gene transcription alone but potentiation of DEX-induced GR-mediated gene transcription). Moreover, the blockade of GR-mediated gene transcription after pretreatment with DMI is consistent with the inhibited CAT gene expression seen up to 8 hr after heat or chemical shock (after which potentiation is observed) (35). It is important to emphasize, however, that there was no evidence that DMI induced chemical shock in the cells. For example, it is known that heat and chemical shock increase proteolysis and decrease protein synthesis (32), yet there was no change in the overall protein content or cellular morphology after DMI treatment. Nevertheless, the GR/hsp complex remains a conceivable site for DMI action.

Interestingly, recent work on the mechanism of action of antidepressants suggests that cAMP and protein kinase A play important roles as mediators of the psychotropic effects of these agents. For example, increased activation of adenylate cyclase (23), nuclear translocation of the cAMP-dependent protein kinase, and increased concentrations of cAMP response element binding protein (36) have been found in the brains of animals after chronic antidepressant administration. Moreover, pertinent to the acute in vitro effects of DMI on GR function, Chen and Rasenick (23) recently reported increased basal levels of cAMP in C6 glioma cells after 1–5 days of DMI (10 μM) treatment. The mechanism of this DMI effect is believed to involve increased functional coupling between G proteins and adenyl cyclase (23). Finally, depressed patients were found to have reduced G protein function in mononuclear cells (37) and reduced cAMP-dependent protein kinase activity in cultured fibroblasts (38).

These findings are particularly intriguing in view of the fact that phosphorylation of the GR and/or other nuclear substrates by cAMP-dependent protein kinase could have a relevant role in the regulation of GR function. For example, much like DMI, both adenylate cyclase and protein kinase A activators have been found to increase GR-mediated gene transcription (39).

Finally, at the concentrations used in this study, DMI binds to several G protein-coupled receptors (e.g., 5-hydroxytryptamine1A, 5-hydroxytryptamine2A, histamine1, α1-adrenergic, α2-adrenergic, muscarinic) (40). It is unclear whether these receptors are expressed by L929 cells. Future studies are necessary to determine whether structurally related or unrelated antidepressants exert similar effects.

In conclusion, we suggest that one important aspect of the
effects of antidepressants may be to facilitate GR-mediated feedback inhibition, by facilitating GR translocation and function, and thereby reverse glucocorticoid hypoperfusion in depression.

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References


