

Differential Effects of Antidepressants on Glucocorticoid Receptors in Human Primary Blood Cells and Human Monocytic U-937 Cells

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A number of data support the assumption that antidepressants (ADs) normalize the altered function of the hypothalamic–pituitary–adrenocortical (HPA) system involved in the pathophysiology of depressive disorder via direct effects on glucocorticoid receptors (GRs). In the present study, we examined the tricyclic ADs desipramine (DESI) and imipramine (IMI), the noradrenaline reuptake inhibitor maprotiline (MAPRO), and the noradrenergic and specific serotonergic AD (NaSSA) mirtazapine (MIR) for their effects on GR expression in primary human leukocytes and in monocytic U-937 cells. Semiquantitative RT-PCR indicated that the ADs exert differential effects on GR-mRNA levels in both primary human leukocytes and U-937 cells: whereas MAPRO and IMI did not induce pronounced changes in GR-mRNA levels, DESI and MIR significantly decreased the amounts of GR-mRNA in both cell systems. Further characterization of the effects of MIR revealed a time dependency of the regulation with an initial increase of GR-mRNA levels above control levels after 2.5 h of treatment and a decrease after 4, 24, and 48 h of incubation. A dose–response analysis demonstrated maximal effects of MIR at a concentration of 10^{-7} M. Immunohistochemical studies showed that MIR increased the GR protein levels in a time-dependent manner and that this upregulation appeared earlier by additional treatment with dexamethasone (DEX). A translocation of the GR protein from the cytoplasm to the nucleus was induced between 24 and 48 h of treatment with MIR and MIR/DEX, respectively. Taken together, our data further support the assumption that ADs influence the neuroendocrine and immune system via effects on cellular GRs.

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INTRODUCTION

Research of the last years fostered the hypothesis that glucocorticoid receptor (GR) dysfunctions are critically involved in the hyperactivity of the hypothalamic–pituitary–adrenocortical (HPA) system present in major depressive disorder (MDD) (Arborelius *et al*, 1999; Holsboer, 2000; Pariante and Miller, 2001). Moreover, it has been shown that the level of the HPA activity in depressive patients is crucially related to the psychopathological course of the disease (Ribeiro *et al*, 1993; Holsboer, 1995; Zobel *et al*, 1999).

Therefore, the effects of antidepressants (ADs) on GRs, the HPA system and its functions are of increasing interest

with regard to their therapeutic mechanism of action. For example, recent research demonstrated that various ADs inhibit the corticosterone-induced gene transcription in a concentration- and time-dependent manner in fibroblast cells (Budziszewska *et al*, 2000). Moreover, long-term treatment of rats with the ADs desipramine (DESI) and amitriptyline (AMI) was found to upregulate hippocampal GR-mRNA levels (Barden *et al*, 1995; Okugawa *et al*, 1999).

The majority of studies using tricyclic ADs, such as DESI, AMI, and imipramine (IMI) indicated an AD-induced GR-upregulation in the brain (Pepin *et al*, 1992; Seckl and Fink, 1992; Reul *et al*, 1993; Przegalinski and Budziszewska, 1993; Budziszewska *et al*, 1994; Pariante and Miller, 2001), whereas only a few studies showed a downregulation of GR in the brain (Brady *et al*, 1991; Biagini *et al*, 1993; Peeters *et al*, 1994; Pariante and Miller, 2001).

In addition to the well-known neuropharmacological actions of ADs and their effects on the neuroendocrine system via GRs, studies have elucidated that the effects of ADs on immune functions may play an important role in the treatment of MDD (Maes *et al*, 1995; Rothermundt *et al*, 2001). Interestingly, functional experiments showed an

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increased resistance of immune cells from depressed patients to the inhibitory effects of glucocorticoids (Pariante *et al*, 1995). Therefore, the neuroendocrine and the immunological data support the hypothesis that the effects of ADs on the properties of GRs and on the GR-induced intracellular signal transduction may represent one of their most important modes of action (Barden *et al*, 1995; Pariante *et al*, 1995; Pariante and Miller, 2001).

Until now, the effects of ADs on the GR have mainly been characterized in *in vitro* systems and in rodents (Pepin *et al*, 1989, 1992; Reul *et al*, 1994; Pariante and Miller, 2001). Therefore, the transfer of the knowledge to the human system is as yet rather limited. Recent data from our group show that ADs are indeed able to upregulate GR-mRNA levels in human peripheral blood cells (Vedder *et al*, 1999), suggesting that such cells may serve as a model system for the study of the effects of ADs on GRs in general.

To further clarify this type of actions in a human system, we here examined the classical ADs DESI, IMI, maprotiline (MAPRO), and the new-class AD mirtazapine (MIR) for their effects on GR-mRNA levels in human primary blood cells of male volunteers and in human monocytic U-937 cells. The distinct pharmacological profile of the AD MIR was the reason for a more detailed characterization of the time- and dose-dependence of its effects on the GR-mRNA in undifferentiated and differentiated human monocytic U-937 cells, respectively. Besides the effects of ADs on the mRNA levels, we investigated GR expression and translocation—important processes involved in the cellular actions of the GR—after MIR and simultaneous treatment with MIR and dexamethasone (DEX) with an immunostaining procedure.

METHODS

Blood Cell Preparations

A total of 11 healthy subjects were screened by a psychiatrist to assure the absence of physical diseases, a history of psychiatric disorders, and the use of any medication. Citrate blood was obtained by venipuncture in the morning between 8.00 and 9.00 am and incubated for 24 h at 37°C on a rotatory roller with 10⁻⁷ M of ADs (DESI, IMI, MAPRO, MIR) dissolved in ethanol and the respective controls with 10⁻⁵ M ethanol. After the incubation, whole blood samples were collected and erythrocytes lysed with 0.8% ammonium chloride. At the end of the lysis, the cell pellets were exposed to 1 ml of Trizol[®] reagent (GibcoBRL, Karlsruhe, Germany) and frozen at -20°C.

U-937 Cells and Culture Conditions

U-937 cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) (Gibco/BRL and Seromed, Berlin, Germany), 0.5% glutamine, and 1% gentamycin at 37°C in a 5% CO₂ atmosphere. For further studies, cells were plated at a concentration of 3 × 10⁵ cells/ml in six-well culture plates (Greiner, Frickenhausen, Germany) and treated with DESI, IMI, MAPRO, and MIR dissolved in ethanol as well as with ethanol-controls (ETH/CON).

ADs were purchased from Sigma-Aldrich (Deisenhofen, Germany); MIR was a kind gift of Organon, Munich,

Germany. The stock concentrations of the ADs in ethanol (10⁻² M) were diluted to the final concentrations of 10⁻⁷ M, and the final concentration of ethanol was 10⁻⁵ M in the treatment samples.

U-937 cells were grown in medium under basal conditions and differentiated to monocytes by treatment with 10 nM phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, Germany) in medium for 3 days. For further studies on the mechanisms of the AD effects, cells were exposed simultaneously to the RNA synthesis inhibitor Actinomycin D (Sigma-Aldrich, Germany) and the ADs (10⁻⁷ M) for 24 h. At the end of the incubation period, the cells were collected and frozen at -20°C.

RNA Extraction

RNA was extracted from the blood cells and the U-937 cells using Trizol[®] reagent in accordance with the manufacturer's instructions. The amount of extracted total RNA was quantified by established optical methods at A₂₆₀/A₂₈₀ (Genequant II, Pharmacia Biotech, Freiburg, Germany) and the structural integrity checked by agarose gel electrophoresis (1.0% agarose (Gibco/BRL)).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

RT-PCR was used to analyze the transcription of the GR- and the house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-mRNA. Total RNA of 1 µg was reverse-transcribed and amplified with the Titan[™] One Tube RT-PCR System (Boehringer, Mannheim, Germany) or reverse-transcribed with 40 U of Superscript II (Gibco/BRL) and 1 µg oligo-(dT) in a total volume of 20 µl following the manufacturer's protocol.

The following primer pairs were used to amplify the cDNA's:

hGR (5' primer: 5'-TGG TGT CAC TGT TGG AGG TTA-3'; 3' primer: 5'-GGC TCT TCA GAC CGT CCT T-3') (453 bp);
GAPDH (5' primer: 5'-CGT CTT CAC CAC CAT GGA GA-3'; 3' primer: 5'-CGG CCA TCA CGC CAC AGT TT-3') (300 bp).

Aliquots of 1 µl cDNA were amplified with a PCR cyclor (Biometra Trio, Göttingen, Germany) for GR and GAPDH using the primers described above with the following cycling program: denaturation for 45 s at 95°C, annealing for 60 s at 59°C, and extension for 60 s at 72°C. PCR products were analyzed for GR (28 cycles) and GAPDH (28 cycles) after amplification and gel electrophoresis in 1% agarose gels. Semiquantitative determination was achieved by digitization of gels with a Polaroid video system (Rothaar & Schroeder, Heidelberg, Germany) and further densitometric evaluation with the Gelscan 4.0 Professional Program (LTF/BioSciTec, Landau/Frankfurt, Germany).

DNA Sequence Analysis

For sequencing, PCR amplification products were gel-purified (QIAGEN, QIAquick Gel Extraction Kit; Qiagen, Hilden, Germany). Direct sequencing of PCR products was performed by cycle sequencing in both directions with ABI PRISM Big Dye Terminator chemistry (Perkin-Elmer, Weiterstadt, Germany) followed by electrophoresis on a

Perkin-Elmer ABI-377 automated sequencer. The amplified GR sequence and the gene-bank information on the GR revealed an identity of 100% of both sequences.

GR Immunofluorescence Detection

For the immunofluorescence experiments, differentiated U-937 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco/BRL) supplemented with 10% heat-inactivated FCS, 2 mM glutamine, and 50 U/ml penicillin and 50 µg/ml streptomycin at 37°C in a 5% CO₂ atmosphere. For further studies, cells were plated and grown in DMEM supplemented with 1% FCS at a concentration of 3×10^5 cells/ml in six-well culture plates (Greiner, Frickenhausen, Germany) and exposed to 10^{-6} M of MIR alone and simultaneously with 10^{-6} M DEX for 1, 2.5, 4, 24, and 48 h. At the end of the incubation period, cells were washed with phosphate-buffered saline (PBS) pH 7.4. Afterwards, cells were permeabilized and fixed with acetone/methanol (1:1) for 15–30 min at 4°C. The fixed cells were washed 4 times with PBS and visualization of intracellular GR expression was carried out by indirect immunofluorescence employing a mouse anti-human GR antibody (H-300) (1:1000) at incubation conditions of 120 min and 4°C (Santa Cruz Biotechnology, Heidelberg, Germany), and a secondary fluorescein isothiocyanate (FITC) labeled rabbit anti-mouse IgG antibody (1:500) for 60 min at 4°C (Dako A/S, Hamburg, Germany). After antibody incubation, the fixed cells were washed 4 times with PBS. For documentation, the photographic equipment of an Axiophot microscope (Zeiss, Jena, Germany), a Spot 32 camera, and a documentation program (Diagnostic

Instruments Zeiss) was used. The quantitative analysis of fluorescence was performed in blindly selected regions from the cytoplasm and the nucleus by densitometric evaluation with the Gelscan 4.0 Professional Program.

Statistical Analysis

Data of different experiments were adjusted with regard to the mean of the respective control group and are presented as mean \pm SEM. If a homogeneity of variances and a normal distribution of data was detectable, one-way analysis of variance and appropriate *post hoc* tests (Student–Newman–Keuls tests) were used for statistical analysis. Values were considered as significant with a $p \leq 0.05$ ($*p \leq 0.05$, $**p \leq 0.01$). If data lacked a homogeneity of variances and/or a normal distribution, they were analyzed with the Kruskal–Wallis one-way analysis of variance on ranks. When this test revealed a significance of the treatment condition, Mann–Whitney rank sum tests were used for between-group comparisons.

RESULTS

Effects of ADs on GR-mRNA Levels in Human Peripheral Blood Cells

One aim of the study was to determine the differential effects of several types of ADs on GR-mRNA transcription in primary human blood cells. After a 24 h treatment, the ADs DESI, IMI, MAPRO, and MIR exerted differential effects on GR-mRNA levels (Figure 1). Semiquantitative evaluation of PCR data indicated that a concentration of

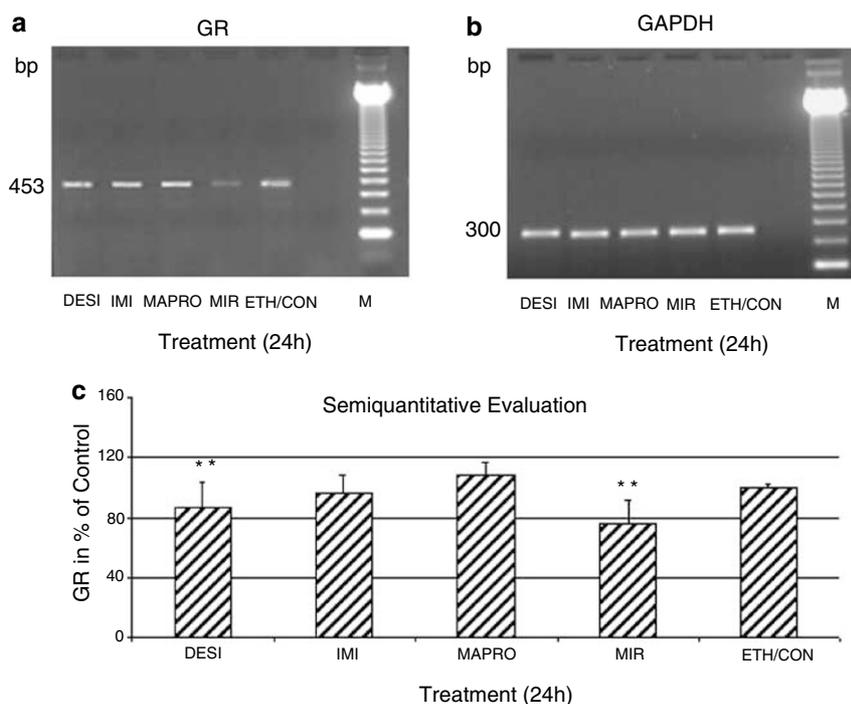


Figure 1 Effects of different ADs (10^{-7} M) on (a) GR and (b) GAPDH-mRNA levels in primary leukocytes after 24 h of treatment. The semiquantitative evaluation for GR-mRNA levels from 11 independent experiments is shown in (c) with a significant downregulation after DESI and MIR treatments (Mann–Whitney rank sum tests for DESI and MIR: $p = 0.006$) (DESI = desipramine; IMI = imipramine, MAPRO = maprotiline, MIR = mirtazapine, ETH/CON = ethanol control).

10^{-7} M of IMI and MAPRO induced only small effects on GR-mRNA levels (96 ± 12 and $108 \pm 9\%$), whereas DESI and MIR significantly downregulated the GR-mRNA levels to 87 ± 16 and $76 \pm 16\%$, respectively (Kruskal–Wallis one-way analysis of variance on ranks: $F = 7.882$, $df = 441.639$, $p < 0.001$; Mann–Whitney rank sum tests for DESI and MIR: $p = 0.006$) (Figures 1a, c). Control experiments showed that these treatments did not induce changes in GAPDH-mRNA levels (Figure 1b).

Effects of ADs on GR-mRNA Levels in Monocytic U-937 Cells

The effects of a 24 h treatment with DESI, IMI, MAPRO, and MIR on GR transcription in undifferentiated U-937 cells are shown in Figures 2a and c. A concentration of 10^{-7} M of DESI and MIR significantly decreased the amounts of GR-mRNA to 60 ± 7 and to $45 \pm 8\%$ of the control levels after 24 h of treatment, respectively (Kruskal–Wallis one-way analysis of variances on ranks: $F = 13.477$, $df = 141.254$, $p \leq 0.001$; Student–Newman–Keuls tests for DESI ($p = 0.002$) and MIR ($p = 0.001$)), whereas IMI did not induce significant changes ($92 \pm 6\%$) ($p > 0.05$). Treatment with MAPRO increased the GR-mRNA levels to $118 \pm 20\%$ of control levels under these conditions, although not to a significant extent ($p > 0.05$) (Figures 2a, c). All treatments did not result in changes of GAPDH-mRNA levels (Figure 2b).

Effects of Actinomycin D Treatment on the AD-Induced Changes in GR-mRNA Levels in U-937 Cells

The nature of the transcriptional modulation of the AD-induced changes was further examined in monocytic cells in

the presence and in the absence of the RNA synthesis inhibitor Actinomycin D (Figure 3). The treatment with Actinomycin D effectively inhibited the biosynthesis of GR-mRNA and GAPDH-mRNA: GR-mRNA levels were reduced to $24 \pm 5\%$ (ETH), $22 \pm 4\%$ (DESI), and $15 \pm 5\%$ (IMI), resulting in an approximately four-fold decrease under treatment conditions with Actinomycin D. Interestingly, GR-mRNA levels after treatment with MAPRO were decreased approximately five-fold from 118 ± 20 to $23 \pm 4\%$, with DESI approximately three-fold from 60 ± 7 to $22 \pm 4\%$, and with MIR only about 0.3-fold from 45 ± 8 to $31 \pm 4\%$, suggesting differential effects of the ADs on GR-mRNA biosynthesis (Figure 3b, lanes 1–10, Figure 3c).

Regarding the GAPDH-mRNA levels, experiments with ADs and Actinomycin D revealed an approximately four-fold decrease without differential effects of ADs (Figure 3b, lanes 1–10, Figure 3c), suggesting a specificity of the effects of the ADs on GR-mRNA levels.

Dose Dependence of the Effects of MIR on GR-mRNA Levels in Undifferentiated U-937 Cells

Investigation of the dose dependence of the effects of MIR on GR-mRNA levels in U-937 cells was done with 10^{-5} , 10^{-6} , and 10^{-7} M of MIR. Figure 4 demonstrates that the different concentrations induce a dose-related manner after treatment for 24 h. Data from the different concentrations (10^{-5} M ETH/CON: $174 \pm 46\%$; 10^{-7} M MIR: $129 \pm 23\%$; 10^{-4} M ETH/CON: $111 \pm 14\%$; 10^{-6} M MIR: $92 \pm 15\%$; 10^{-3} M ETH/CON: $100 \pm 5\%$; 10^{-5} M MIR: $71 \pm 13\%$) were normalized with regard to the 10^{-5} M ETH/CON treatment (one-way analysis of variances on ranks: $F = 3.786$, $df = 173.097$, $p = 0.027$).

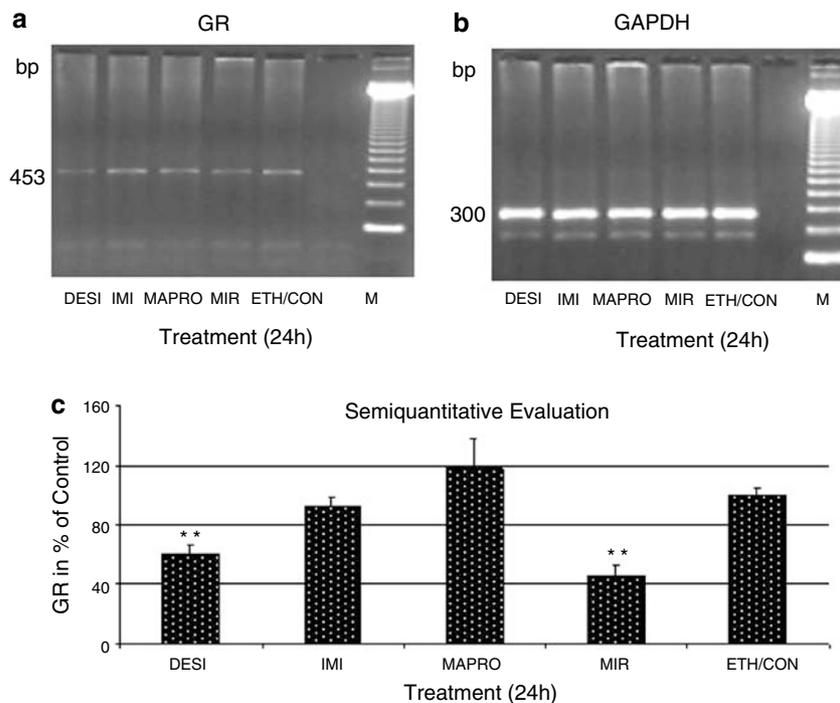


Figure 2 Effects of different ADs (10^{-7} M) on GR-mRNA levels in U-937 cells after treatment for 24 h. The PCR data are shown for (a) GR and (b) GAPDH-mRNA levels. A semiquantitative evaluation for GR-mRNA from six independent experiments is shown in (c). Statistical evaluation of data revealed a significance of changes in GR-mRNA levels after treatment with DESI ($p = 0.002$) and MIR ($p = 0.001$) Student–Newman–Keuls tests.

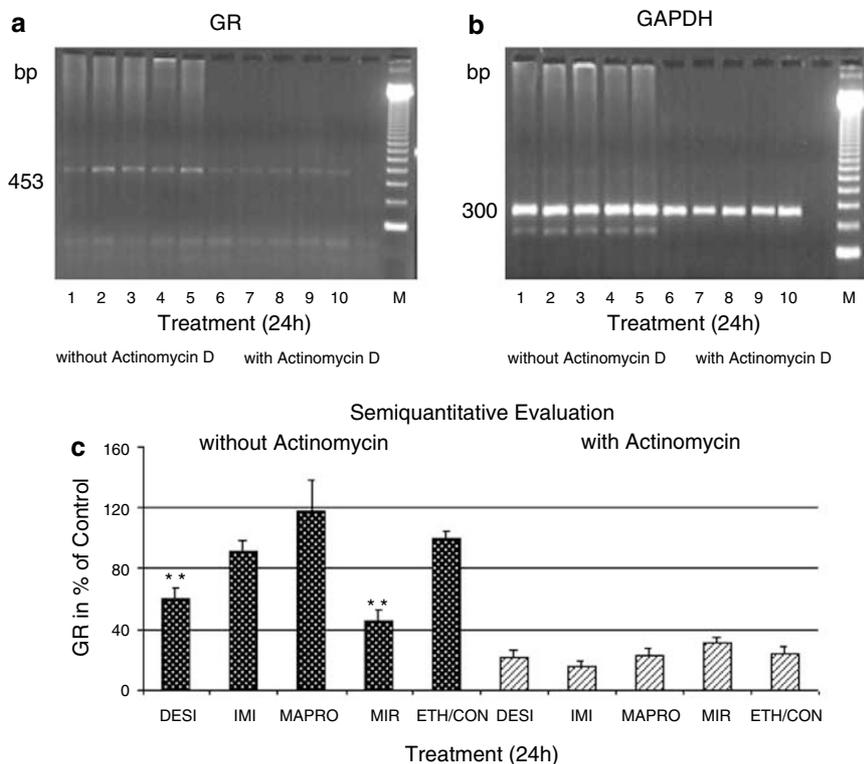


Figure 3 Actinomycin D induces a decrease in RNA amplification of GR- and GAPDH-mRNA levels after 24 h of treatment in U-937 cells (a (6–10) and b (6–10)). A semiquantitative evaluation of Actinomycin D treatment compared to untreated U-937 cells is derived from three independent experiments and shown in (c). Statistical evaluation of data revealed a significant decrease of GR-mRNA levels after MIR and DESI treatment (Mann–Whitney rank sum tests for DESI and MIR: $p = 0.006$) (1–5: 1 = DESI, 2 = IMI, 3 = MAPRO, 4 = MIR, 5 = ETH/CON; 6–10: 1–5+Actinomycin D at each treatment point).

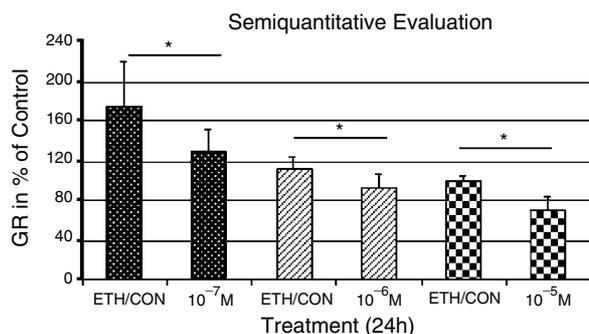


Figure 4 Dose-related effects of MIR and ETH on GR-mRNA levels after 24 h of treatment in undifferentiated U-937 cells. All three dosages of MIR (10^{-5} , 10^{-6} , 10^{-7} M) and ETH (10^{-3} , 10^{-4} , 10^{-5} M) affect GR-mRNA levels in U-937 cells. The maximal effect of MIR on the GR-mRNA was shown after a concentration of 10^{-7} M. A semiquantitative evaluation for GR-mRNA from three independent experiments is shown. Statistical evaluation of data revealed a significance of changes in GR-mRNA levels after treatment with MIR ($p = 0.027$) (Mann–Whitney rank sum tests).

The most pronounced effect of GR-mRNA downregulation was shown after 10^{-7} M MIR treatment. Subsequently, this concentration was used for the additional experiments.

Time-Dependent Effects of MIR on GR-mRNA Levels in Undifferentiated and Differentiated U-937 Cells

Ragg *et al* (1998) induced U-937 cells to differentiate by a treatment with 40 nM PMA. This treatment differentiates

U-937 cells to nondividing adherent monocytic cells. We obtained the same morphological changes after treatment with only 10 nM PMA. The morphology of the undifferentiated and differentiated U-937 cells is shown in Figures 5a and b.

MIR-induced changes in GR-mRNA levels were further characterized after treatment of U-937 cells in a time-dependent manner. Data suggest that a concentration of 10^{-7} M MIR exerts differential time-dependent effects on GR-mRNA levels with an initial upregulation from 103 ± 10 to $129 \pm 44\%$ after 2.5 h of treatment (1 h: 100 ± 4 to $105 \pm 14\%$). A downregulation of GR-mRNA levels from 124 ± 27 to $85 \pm 14\%$ after 4 h, from 125 ± 32 to $98 \pm 21\%$ after 24 h, and from 120 ± 6 to $83 \pm 14\%$ after 48 h was detectable (Figures 5c, e).

Because PMA induced a differentiation of the morphology of U-937 cells, we also investigated the possible changes of GR-mRNA levels after simultaneous treatment with PMA and MIR.

PCR and the densitometric quantification data are shown after PMA-induced differentiation in Figures 5d and f. The results indicate small effects of MIR with downregulation of GR-mRNA levels after 1 h from 100 ± 4 to $88 \pm 10\%$ and a normalization after 48 h of treatment (100 ± 3 to $105 \pm 5\%$) (Figures 5d, f). After 2.5 h of MIR treatment, an upregulation of GR-mRNA from 105 ± 3 to $119 \pm 8\%$ occurred, whereas after 4 and 24 h of treatment only marginal changes were detectable from 109 ± 4 to $100 \pm 5\%$ and from 109 ± 4 to $95 \pm 9\%$. Overall, the induced changes in the amounts of GR-mRNA did not reach the significance level

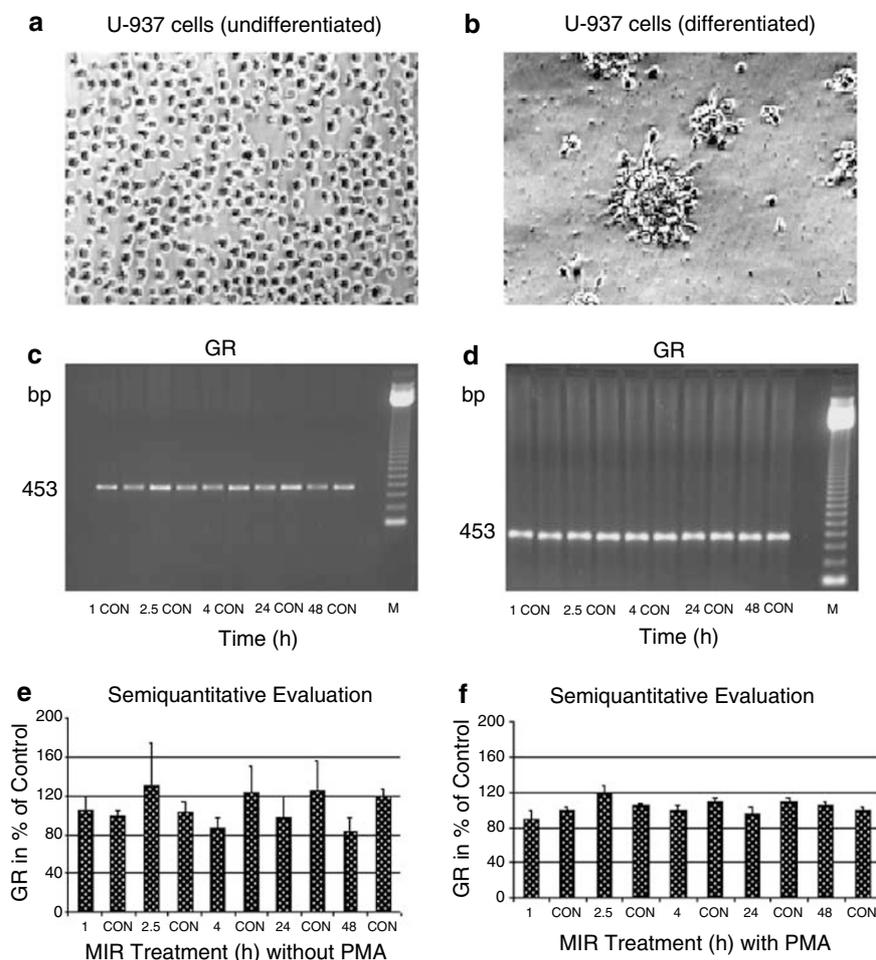


Figure 5 Time-related effects of MIR on GR-mRNA in undifferentiated and differentiated U-937 cells. Morphology of undifferentiated and differentiated U-937 cells is shown in (a) and (b). For differentiation, the cells were cultured for 72 h after the addition of 10 nM PMA. *Undifferentiated U-937 cells*: The PCR amplification data (c) and semiquantitative analysis of PCR data (e) from three independent experiments show the effects of MIR in undifferentiated U-937 cells after different treatment periods (1, 2.5, 4, 24, and 48 h) in comparison to the respective solvent-treated control. *Differentiated U-937 cells*: The PCR amplification data (d) and semiquantitative analysis of PCR data (f) from three independent experiments demonstrate the effects of MIR in differentiated U-937 cells after different treatment periods (1, 2.5, 4, 24, and 48 h) in comparison to the respective solvent-treated control.

and were markedly less pronounced in differentiated cells (Figures 5d, f) if compared to the undifferentiated conditions (Figures 4c, e).

Effects of MIR and MIR/DEX on GR Protein Expression in U-937 Cells

To examine the differential effects of MIR and MIR/DEX on GR protein expression, we used the fluorescence/immunostaining method in differentiated U-937 cells.

Since basal expression and translocation of GRs are regulated by glucocorticoids and an interaction between ADs and glucocorticoids is likely, effects were also examined under additional treatment with the glucocorticoid DEX. Cells were treated with 10^{-6} M MIR or with 10^{-6} M of both MIR and DEX, and the effects on GR expression were examined in a time-dependent manner after 1, 2.5, 4, 24, and 48 h. Initially, the pattern of staining was heterogeneous among the cells with a diffuse fluorescence in both the cytoplasm and the nucleus. In most of the cells, the fluorescence was more intense in the cytoplasm

than in the nucleus, and a continuous increase of GR protein was detectable between 4 and 48 h of MIR treatment (Figures 6g, i, k). The simultaneous treatment with MIR and DEX enhanced the GR protein expression after 1 and 2.5 h if compared to the MIR treatment alone (Figures 6d, f). After 48 h of MIR (MIR/DEX) treatment, GR levels were significantly increased to $119 \pm 11\%$ ($139 \pm 9\%$) in the cytoplasm and to $231 \pm 23\%$ ($231 \pm 25\%$) in the nucleus (Figure 7). Cells grown under AD- and DEX-free conditions showed a weak cytoplasmic GR expression for all time points (demonstrated in the figures only for the 48 h treatment point), whereas the GR protein was not or only barely detectable in the nucleus under both ETH treatment conditions (Figures 6a, b).

Effects of MIR and MIR/DEX Treatment on the GR Translocation in U-937 Cells

For a better characterization of the GR translocation in U-937 cells after MIR and MIR/DEX treatment, we chose the time points of 1 and 48 h. After 1 h of treatment with both

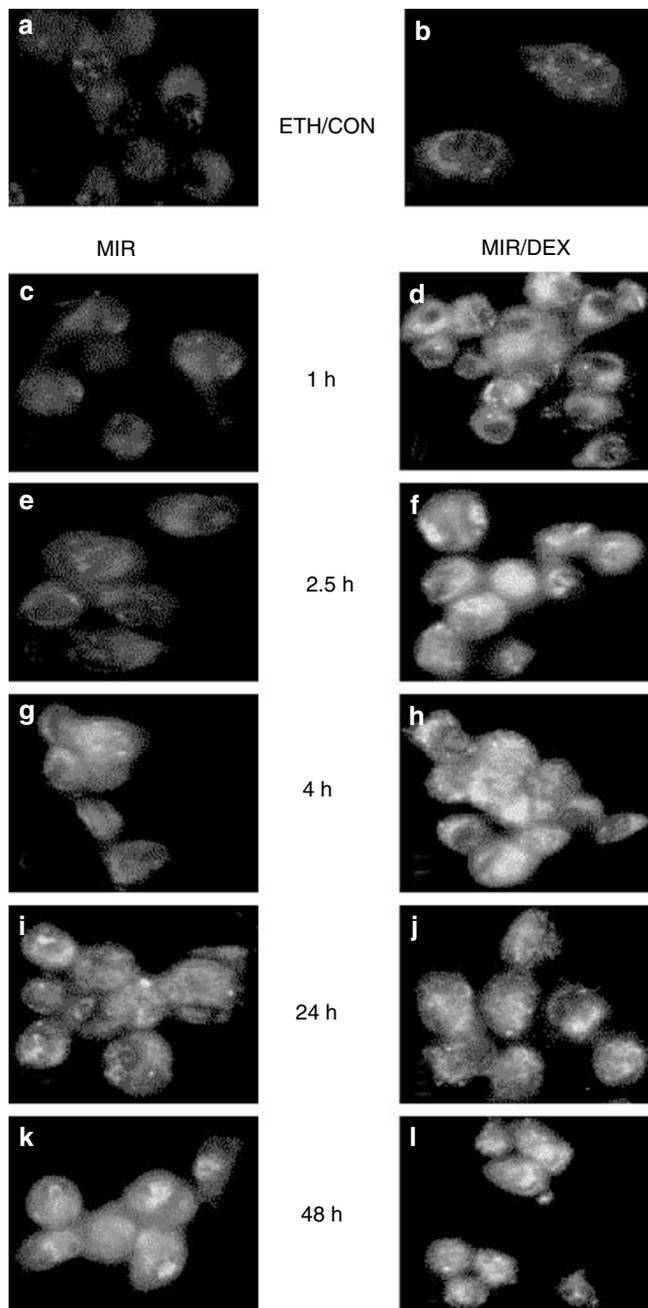


Figure 6 Time-related effects of MIR and MIR (10^{-6} M)/DEX(10^{-6} M) on GR protein expression and translocation from the cytoplasm to the nucleus from three independent experiments as determined by immunostaining in U-937 cells. Cells were differentiated to nondividing adherent cells with 10 nM PMA and grown under control conditions (ETH/CON) for 48 h (a, b), with MIR (10^{-6} M) and [MIR/DEX] for 1 h (c)[d], 2.5 h (e)[f], 4 h (g)[h], 24 h (i)[j], and 48 h (k)[l]. The fluorescence was more intense in the cytoplasm than in the nucleus and an initial increase of GR protein in the cytoplasm was obvious after 4 h of MIR treatment (g) and after 1–2.5 h of MIR/DEX treatment (d). The GR protein levels in the nucleus were increased after 48 h if compared to levels in the cytoplasm (k, l). After 48 h, cells grown under AD- and DEX-free conditions showed only a weak cytoplasmatic GR protein expression (a, b).

MIR and MIR/DEX, the cytoplasm was more intensely stained than the nucleus (Figures 7a, b). Moreover, treatment with the combination of MIR and DEX (Figure

7b) led to a more intense staining in the cytoplasm if compared to MIR treatment alone (Figure 7a). After 48 h of treatment, a translocation of the GR protein from the cytoplasm to the nucleus with increased levels of GR protein in the nucleus was detectable in at least 50% of cells under both conditions (MIR and MIR/DEX) (Figures 7c, d).

Quantitative analysis of fluorescence was performed in blindly selected regions of the cytoplasm and nucleus by densitometric evaluation with the Gelscan 4.0 Professional Program. The results were standardized with regard to the levels of GR immunoreactivity in the cytoplasm (MIR, 1 h time point) and reached $100 \pm 10\%$ in this cellular compartment and $81 \pm 12\%$ in the nucleus (Figures 7a, e). After 48 h of MIR treatment, GR levels were significantly increased to $119 \pm 11\%$ in the cytoplasm and to $231 \pm 23\%$ in the nucleus (Figures 7c, e). Evaluation of data using the Kruskal–Wallis one-way analysis of variance on ranks showed a significance of the treatment ($H = 29.151$, $df = 7$, $p < 0.001$).

Statistical analysis of data in the cytoplasm (a) and the nucleus (b) after 1 h of MIR treatment showed significant changes in comparison to the data of the cytoplasm and the nucleus after 48 h MIR treatment (a): ($p = 0.008$, $p \leq 0.001$), (b): ($p = 0.002$, $p \leq 0.001$).

Similar effects were detectable for a concomitant treatment with MIR and DEX resulting in increased GR protein levels after 1 h of treatment in the cytoplasm ($122 \pm 8\%$) and a decrease in the nucleus ($79 \pm 7\%$) (Figures 7b, e). After 48 h of MIR/DEX treatment, the GR protein levels in the cytoplasm were increased to $139 \pm 9\%$ and to $231 \pm 25\%$ in the nucleus (Figures 7d, e).

Statistical comparison of data from the cytoplasm (a) and the nucleus (b) after 1 h MIR/DEX treatment with data from the cytoplasm and the nucleus after 48 h MIR/DEX treatment also showed a significance of treatments: (a) ($p = 0.02$, $p \leq 0.001$); (b) ($p \leq 0.05$, $p \leq 0.001$).

DISCUSSION

Despite the increasing evidence that supports the assumption of a disturbed GR function in depressive patients (Holsboer, 1995, 2000; Modell *et al*, 1997; Pariante and Miller, 2001), only a few studies, including our own (Vedder *et al*, 1999), exist that conclusively point to direct effects of ADs on the regulation of GR-mRNA levels in the human system.

Therefore, we have extended our initial approach to further support the hypothesis of a modulation of GR-mRNA levels by ADs in human blood cells. In our previous study, we have shown that both AMI (a classical tricyclic AD) and DEX (a synthetic glucocorticoid with antidepressive actions) (Arana *et al*, 1995) increase the GR-mRNA levels in human primary leukocytes (Vedder *et al*, 1999). In the present study, we chose ADs with different pharmacological properties such as DESI, IMI, MAPRO, and MIR, an AD with a distinct mechanism of combined noradrenergic and serotonergic effects to characterize their actions on human primary leukocytes and on the U-937 monocytic cell line in more detail.

Our data show that in contrast to MAPRO and IMI, the ADs DESI and MIR significantly decrease GR-mRNA

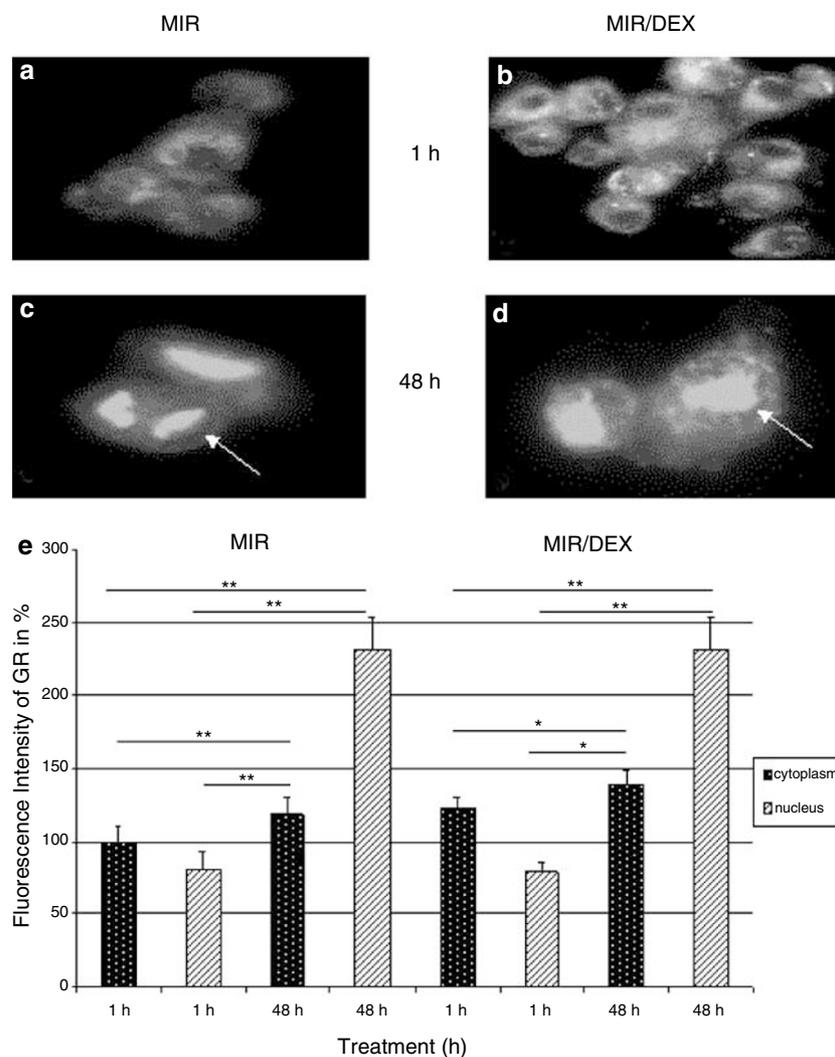


Figure 7 GR protein expression and translocation after MIR and simultaneous MIR and DEX treatment in U-937 cells. After treatment with MIR and MIR/DEX for 1 h, the cytoplasm was more intensely stained than the nucleus (a, b). After 48 h of treatment with MIR and MIR/DEX, a distinct translocation of GR protein from the cytoplasm to the nucleus was found in the cells (c, d). Quantitative analysis of fluorescence from the cytoplasm and nucleus is demonstrated in (e). Statistical evaluation of data revealed a significance of changes in GR protein between the cytoplasm and the nucleus (Mann–Whitney rank sum test: * $p \leq 0.05$, ** $p \leq 0.01$) (e).

levels after 24 h of incubation in both models. Our data of a downregulation of GR-mRNA after DESI treatment are not in line with the data of Pariante *et al* (1997). Only the short-term treatment (2.5 h) with MIR leads to an increase in GR-mRNA levels in undifferentiated U-937 cells, whereas a decrease was detectable after 4, 24, and 48 h of treatment. Such a time dependence of the effects of AD on GR-mRNA levels has also been described under *in vivo* conditions supporting the assumption that the time factor is indeed relevant for the changes induced (Reul *et al*, 1994).

Thus, the general effects of ADs on the GRs are detectable in an important cell population of the immune system, which has not only been shown to display alterations in depression (Maes, 1995; Seidel *et al*, 1996; Lanquillon *et al*, 2000; Rothermundt *et al*, 2001) but has also been demonstrated to be strongly connected to the psychopathological state (Maes *et al*, 1999; Rothermundt *et al*, 2001)

and to the activation of the HPA system in depression, possibly caused in part or completely by proinflammatory cytokines like IL-1 and IL-6 (Maes *et al*, 1993a, b; Lanquillon *et al*, 2000). Moreover, the direct actions of ADs on immune functions such as the cytokine production (Xia *et al*, 1996) may be crucially involved in the treatment response to ADs (Lanquillon *et al*, 2000). With regard to our results, such effects may be caused via the AD actions on the GR-mRNA and on GR-mediated functions in immune cells and probably even specifically leukocytes. The dose–response relationship for the effects of MIR and the studies on the primary human leukocytes further support the relevance of the data. The dosages of MIR and of the other ADs used in our experiments are within the range of clinically relevant concentrations (Dodd *et al*, 2000), and the effects on the primary human leukocytes further validate the results on the monocytic cell line U-937.

Our experiments with Actinomycin D indicate that the regulatory influences of the ADs may be mediated via differential effects of the substances on the biosynthesis of the mRNA rather than other mechanisms such as effects on the stability of the mRNA. DESI and MIR induced a decrease in GR-mRNA levels compared with the other ADs, suggesting a specific effect on the GR, especially if considered in contrast to the GAPDH-mRNA levels.

In addition, our 'control data' on the dose-response and on the time course of the effects of MIR suggest a separate effect of ETH on GR-mRNA levels. The incubation with increasing concentrations of ETH results in a decrease of GR-mRNA levels under our conditions. Such a decrease would be in line with very recent data from the literature showing that acute treatment of human mononuclear cells—similar to the cells used in this study—results in an increase in nuclear GRs and a reduced GR binding to the glucocorticoid response elements (GRE) (Szabo and Mandrekar, 2002). We here show that ETH increases GR-mRNA levels in a dose-dependent manner in our system. Moreover, the studies on the time course of the effects of ETH suggest that the effect occurs firstly after 4 h and is stable up to 48 h in undifferentiated U-937 cells. Other studies revealed that ETH also affected GRE binding under *in vivo* conditions in the rat brain, and that mianserin, another AD, interacts with the effects of ETH on the GR system (Roy *et al*, 2002). In conjunction with these data, we suggest that the effects of MIR—and also other ADs in our system such as DESI—may result, to a certain extent, from an interaction with the effects of ETH.

In addition to the basic data on the regulation on GR-mRNA levels, we examined functional changes of the GR protein in monocytic U-937 cells by an immunohistochemical approach. These experiments revealed that MIR induced the expression of the GR protein and its translocation from the cytoplasm to the nucleus. This represents one of the most important steps in the initiation of the GR-mediated genomic responses (Beato *et al*, 1987, Beato and Klug, 2000) and has been shown for ADs in other systems such as fibroblastic cells (Pepin *et al*, 1992). This study, performed on mouse fibroblasts (LTK cell line) indicated that DESI stimulates the promoter activity of the GR gene after 24 h of treatment and leads to an increase in GR binding after 72 h of treatment. Based on these data, these authors conclude that DESI may directly stimulate the transcription of the GR gene. Pariante *et al* (1997), however, demonstrated that in the absence of steroids, DESI is able to induce a GR translocation without activation of GR-mediated gene transcription in L 929 cells. Additionally, the authors showed that treatment with DESI in the absence of steroids leads to an inhibition of GR gene transcription if compared to the effects in cells treated with DEX alone or after simultaneous application of DESI and DEX.

We extend these data to the human system and show that DESI and MIR downregulate the GR-mRNA levels in human primary leukocytes and in the undifferentiated monocytic cell line U-937. Although MIR downregulated the GR-mRNA after 4, 24, 48 h treatment in undifferentiated U-937 cells, in differentiated cells the GR-mRNA was hardly affected by the treatment at these time points. This result did not correspond to the increase of the GR

protein after 4 h MIR treatment that was shown with immunostaining methods in differentiated U-937 cells and appear earlier (1h) by concomitant incubation with MIR and DEX. These findings of an upregulation of the GR protein after MIR and combined MIR and DEX treatment support the assumption that the effects of ADs are enhanced by glucocorticoids despite a downregulation of the mRNA levels after treatment with MIR in undifferentiated U-937 cells.

The effects of MIR to upregulate the GR-mRNA after short-time treatment (2.5 h) and to downregulate the GR-mRNA after long-time treatment (24–48 h) in undifferentiated cells are likely to be delayed on the basis of GR protein expression. We assume that the upregulation of the GR protein after 4–48 h treatment is followed by a downregulation of the GR protein at a later time. In this case, MIR might stabilize the GR protein half-life time. Additionally, we demonstrate a translocation of the GR protein after MIR and MIR/DEX treatment in a time-dependent manner. Our results show a translocation of the cytoplasmic GR protein to the nucleus after treatment with MIR or with MIR/DEX. The treatment with the AD MIR alone and in the presence of the steroid DEX had no further effect on the staining of the cells or the nucleus. On the other hand, Pariante *et al* (1997) demonstrated an effect of brighter nuclei by concomitant DESI/DEX treatment in comparison to a single treatment with DESI or DEX.

Under our conditions, the addition of DEX led to an enhancement of MIR effects with regard to the GR protein. If it is assumed that ADs need a basal concentration of GR activation by steroids for their effects, the actions of MIR might be mediated or at least influenced by basal steroids, present in the blood or in the media. Further addition of DEX would then lead to an additional—although rather slow—translocation of the GR protein to the nucleus. Regarding the effects of the AD and DEX on GR-mediated signal transduction, interactions with membrane steroid transporters such as the multidrug resistance p-glycoprotein have been shown as a potentially relevant mechanism of action (Pariante *et al*, 2001). It was hypothesized that this would lead to an increase in the intracellular concentrations of steroids and subsequently increase GR-induced gene transcription. Such a mechanism might not only explain the parallel effects of DEX and several ADs, but might also be an explanation for the rather slow effects of DEX under our conditions.

In summary, our data show differential effects of several ADs including the new AD MIR on GR-mRNA induction and effects of MIR on GR protein expression and functional parameters such as nuclear translocation. Although MIR upregulated the GR-mRNA at an early phase (2.5 h), it downregulated the GR-mRNA at a late phase (24–48 h); the GR protein was upregulated at an early phase (4 h), while the translocation of GR from the cytoplasm to the nucleus was induced at a late phase (24–48 h). Thereby, these data show both a downregulation of mRNA levels and a likely increase in GR-mediated signal transduction after treatment with the AD MIR. Overall, our data would support the hypothesis that ADs exert their therapeutical actions in the treatment of depressive disorders via their influence on GRs in the HPA-axis and in the immune system.

ABBREVIATIONS

ADs, antidepressants; CNS, central nervous system; DESI, desipramine; DMEM, Dulbecco's modified Eagle's medium; DEX, dexamethasone; FITC, fluoresceine-isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GRs, glucocorticoid receptors; HPA, hypothalamic pituitary adrenocortical; IMI, imipramine; MAPRO, maprotiline; MDD, major depressive disorder; MIR, mirtazapine; NaSSA, noradrenergic and specific serotonergic antidepressant; PCR, polymerase chain reaction; PMA, phorbol 12 myristate-13 acetate; RNA, ribosomal nucleic acid; RT, reverse transcriptase.

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