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Melatonin Inhibits Glucocorticoid Receptor Nuclear Translocation in Mouse Thymocytes

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The antiapoptotic effect of melatonin (MEL) has been described in several systems. In particular, MEL inhibits glucocorticoid-mediated apoptosis. Our group previously demonstrated that in the thymus, MEL inhibits the release of Cytochrome C from mitochondria and the dexamethasone-dependent increase of *bax* mRNA levels. In this study we analyzed the ability of MEL to regulate the activation of the glucocorticoid receptor (GR) in mouse thymocytes. We found that even though the methoxyindole does not affect the ligand binding capacity of the receptor, it impairs the steroid-dependent nuclear translocation of the GR and also prevents transformation by blocking the dissociation of the 90-kDa heat shock protein. Coincubation of the methoxyindole with dexamethasone did not affect the expression of a reporter gene in GR-transfected Cos-7 cells or HC11 and L929 mouse cell lines that express Mel-1a and retinoid-related orphan receptor-α (RORα) receptors. Therefore, the antagonistic effect of MEL seems to be specific for thymocytes, in a Mel 1a- and RORα-independent manner. In summary, the present results suggest a novel mechanism for the antagonistic action of MEL on GR-mediated effects, which involves the inhibition of 90-kDa heat shock protein dissociation and the cytoplasmic retention of the GR. (*Endocrinology* 147: 5452–5459, 2006)

IT HAS BEEN demonstrated that melatonin (MEL), a natural compound synthesized in a variety of organs, is a cell-protective agent (for a review see Refs 1, 2). The antiapoptotic activity of MEL was previously described in several systems such as immune cells (3–5), cerebellar neurons (6), and rat brain astrocytes (7).

Apoptosis is a complex process controlled by external signals and also an internal genetic program involved in the regulation of homeostasis and the proper functioning of tissues and organs. Several diseases as cancer, autoimmunity, persistent infections, and neurodegeneration have been ascribed to a failure in the apoptotic program (8–10). In this sense, malfunction of the death machinery resulting from the mutations of genes that code for proteins involved in the apoptotic program has been reported, *i.e.* the p53, an executioner of DNA damage triggered apoptosis, and Bax, a proapoptotic molecule with the ability to perturb mitochondrial membrane integrity, are frequently mutated in malignant neoplasms (8). Antiapoptotic proteins like bcl-2, cellular-inhibitor of apoptosis protein 2, and neuronal apoptosis inhibitory protein 1 are often altered in follicular lymphomas (9).

The thymus is a well-known model for apoptosis studies. It has been demonstrated that not only the chronic administration of MEL but also the coincubation of the methoxyindole with dexamethasone (DEX) reduce the glucocorticoid-induced programed cell death in thymocytes (5, 11). In that sense, our group demonstrated that MEL inhibits apoptosis by preventing cytochrome C release and reducing the glucocorticoid mediated-increase in Bax levels.

The mechanisms whereby MEL influences apoptosis have not been clarified yet, although different options have been suggested. There are at least four possible scenarios to explain its biological effects: 1) its antioxidant properties as scavenger of free radicals (12–15) and/or its specific interaction with 2) the plasma membrane receptors (16–19), 3) nuclear receptors (20–23), and 4) Ca^{2+} binding proteins such as calmodulin (24–26). In a previous work (5), we suggested that the antiapoptotic effect of MEL in thymocytes could be due to the interaction of the methoxyindole with specific receptors. The presence of MEL membrane receptors (Mel 1a) has been described in rat T and B lymphocytes (18). On the other hand, it is known that MEL is a natural ligand of retinoid Z receptor (RZR)α and RZRβ, two members of ROR, retinoid-related orphan receptor (ROR) family. Through these receptors, melatonin regulates the expression of several genes, *i.e.* the down-regulation of 5-lipoxygenase expression, mediated by RZRα receptor in B lymphocytes (27, 28). MEL also binds to purified thymocyte nuclei (29), suggesting the existence of functional ROR family receptors in the thymus.

Inhibition of the glucocorticoid receptor-dependent gene expression by MEL was described by Persengiev (30), who reported that the inhibition of glucocorticoid receptor (GR) transcriptional activity of a mouse mammary tumor virus (MMTV) promoter construct was dependent on the coexpression of both MEL receptor Mel 1a and GR.

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Abbreviations: CaM, Ca^{2+} binding protein calmodulin; DEX, dexamethasone; FCS, fetal calf serum; GR, glucocorticoid receptor; GRE, glucocorticoid response element; hsp90, 90-kDa heat shock protein; MEL, melatonin; Mel 1a, MEL membrane receptor; MMTV, mouse mammary tumor virus; PMSF, phenylmethylsulfonyl fluoride; ROR, retinoid-related orphan receptor; RZR, retinoid Z receptor.

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Because MEL is capable of preventing DEX-induced gene expression, it seems possible that the methoxyindole could control the ability of the activated GR to reach their specific target promoters.

The activation of GR involves several structural alterations occurring in its ligand binding domain. These changes are translated to the receptor surface including the DNA binding site. As a consequence, those modifications allow the GR interaction with specific glucocorticoid response elements (GREs) of target genes. Thus, the antagonistic effect of a given compound could affect some of the events related to the ligand/GR interaction and the consequent biological response.

In the absence of hormone, GR is a 90-kDa heat shock protein (hsp90)-regulated transcription factor primarily localized in the cytoplasm. Upon ligand binding, GR rapidly translocates to the nucleus. The retrograde movement of the GR throughout the cytoplasm seems to occur by cytoskeletal tracks, cytoplasmic dynein being the motor protein used for the movement (31). Also, the phosphorylation status of cytoplasmic steroid receptors seems to be relevant for the regulation of their nuclear import and further interaction with the DNA (32–34). It is well known that the association of hsp90 with the GR is a requirement to maintain the receptor not only in its steroid binding conformation but also in a transcriptional inactive state, even when the receptor is located in the nucleus (35). Dissociation of hsp90 from the receptor allows receptor binding to DNA and the subsequent regulation of gene transcription (35). The transcriptional specificity of GR is achieved by interaction of the P-box in the first zinc finger of the DNA-binding domain of GR with a palindromic GRE (36). This type of regulation has been identified in a large variety of genes, including some Bcl-2 family proteins involved in the control of apoptosis, particularly the *bcl-X* gene (37). GR activity is also regulated by factors present in the promoter of the specific DNA sequences to which GR binds (36).

The present paper analyzes the ability of MEL of regulating GR activation. It is described that the methoxyindole prevents GR transformation in thymus by impairing hsp90 dissociation from the receptor and GR nuclear translocation. Intriguingly, MEL was unable to modify GR activity in other cell types such as L929 mouse fibroblasts, HC11 mouse mammary epithelial cells, and Cos-7 cell line, suggesting that this inhibitory effect on the GR is tissue specific.

Materials and Methods

Reagents and hormones

DEX, MEL, concanavalin A, and RPMI 1640 medium were purchased from Sigma (St. Louis, MO). [^{3}H]DEX (75 Ci/mmol) was supplied by NEN Life Science Products (Boston, MA). Fetal calf serum (FCS) and DMEM were provided by Invitrogen (Carlsbad, CA). Hormones were dissolved in absolute ethanol and used at a final concentration of 10 nM. A stock solution of concanavalin A was prepared in distilled water and added to RPMI 1640 medium just before incubations. FCS was delipidated with charcoal dextran as previously described (38).

Animals and thymocytes preparation

CF-1 male mice (21 d old) were housed in a standard animal room with food and water *ad libitum* under controlled conditions of humidity and temperature (21 ± 2 C). Fluorescent lights were turned on automatically every 12 h, from 0600 to 1800 h. All animals were treated and cared in accordance with standard-international animal care protocols (39). Mice were killed by cervical dislocation at fixed hours (between 1100 and 1200 h) to correct for diurnal variations in serum MEL levels. Thymuses were immediately removed and extensively minced with scissors in ice-cold RPMI 1640 medium. The cell suspension was filtered through Nytex and viable cells were counted in a Neubauer hemocytometer in the presence of 0.04% of Trypan-Blue.

In vitro incubation of thymocytes

Thymocytes (10^7 cells/well) were incubated in plastic dishes in 1 ml RPMI 1640 medium containing 10% charcoal-stripped FCS, concanavalin A (2 μg/ml), and 10^{-8} M DEX in the presence or absence of 10^{-8} M MEL. The corresponding volume of ethanol (0.1%) was added to control cells. Cells were incubated at 37 C for different times in a water bath under a normal atmosphere. After incubation, cells were gently resuspended, placed in a 1.5 ml tube, and centrifuged at 2000 rpm for 5 min at room temperature.

Competition assays

CF-1 male mice (21 d old) were adrenalectomized 48 h before experiments and maintained on Purina chow (diet l), saline, and fresh water *ad libitum*. Animals were killed by cervical dislocation. Thymuses were used as a source of GR. Glands were homogenized with two volumes of lysis buffer at pH 7.4 [10 mM HEPES, 5 mM EDTA, 20 mM Na_2MoO_4, 10% glycerol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF)] and centrifuged at 15,000 × *g* for 30 min at 4 C. Supernatants were removed and referred to as cytosol. Protein concentration was quantified according to Bradford (40). Five hundred microliters of cytosol (600 μg protein) were incubated during 16 h at 4 C with 5 nM [^{3}H]DEX and either 5 μM DEX or 5 μM MEL. Bound hormone was separated by adsorption with one volume of charcoal-dextran (2%:0.2%) in PBS (140 mM NaCl, 3 mM KCl, 1.5 mM KH_2PO_4, 16 mM NaH_2PO_4) followed by centrifugation at 3000 × *g* for 15 min. Bound radioactivity was determined in 400 μl supernatant.

Subcellular fractions separation

The 10^7 thymocytes were incubated for 30 min with or without DEX in the presence or absence of 10 nM MEL. For subcellular fraction separation, cells were centrifuged for 5 min at 2000 rpm and washed with ice-cold PBS buffer. Cells were then incubated in 200 μl of hypotonic buffer [10 mM Tris-HCl (pH 6.7); 0.2 mM $MgCl_2$; 1 mM EGTA; 0.05 mM leupeptin; 1 mM PMSF; 1 μM pepstatin A] for 5 min on an ice-water bath and lysed by douncing homogenization. Samples were centrifuged at 1500 rpm for 10 min at 4 C and supernatants were referred as cytoplasmic fraction. Pellets were washed twice in ice-cold PBS buffer, resuspended in 100 μl of lysis buffer [20 mM Tris-HCl (pH 6.7); 70 mM NaCl; 10% glycerol; 1% Triton X-100; 0.5% Nonidet P-40; 300 U/ml DNase I (Sigma); 0.05 mM leupeptin; 1 mM PMSF; 1 μM pepstatin A], and incubated on ice-water bath for 30 min. After the addition of 0.5 M NaCl, the incubation was continued for additional 30 min. Samples were centrifuged at 12000 rpm for 5 min at 4 C, and supernatants were referred to as nuclear fraction.

Confocal microscopy

For indirect immunofluorescence studies, 10^4 thymocytes or HC11 cells were incubated for 30 min at 37 C with either 10 nM DEX or 10 nM MEL or both hormones together. Then a drop of the thymocytes suspension was extended on a glass cover slide that was waved over a flame for 5 sec. Thymocytes and HC11 cells were finally fixed with 4% paraformaldehyde in PBS for 30 min at room temperature. Cells were permeabilized with 0.1% sodium dodecyl sulfate in PBS for 5 min and washed three times with PBS. Cells were blocked in PBS containing 3% BSA (PBS-BSA) for 30 min at room temperature and then incubated for 2 h at room temperature with an anti-GR antibody (BuGR2 clone, Affinity Bioreagents, Golden, CO) at 1:50 dilution in PBS-BSA. Cells were washed with PBS-BSA and incubated for 30 min with Cy2-conjugated secondary antibody antimouse IgG (492 nm excitation wavelength and 510 nm emission wavelength, catalog no. 711-225-152, Jackson Immu-

noResearch, West Grove, PA) diluted 1:200 in PBS-BSA. Cells were washed three times with PBS, incubated with 0.5 μg/ml propidium iodide for 5 min, and washed three times with PBS and once with distilled water for 5 min. Finally, cells were mounted on a glass slide by adding a drop of 50% glycerol in PBS. Fluorescence was detected with a FV300 laser scanning microscope (Olympus America Inc., Melville, NY) and images were analyzed with FluoView software (Olympus).

Coimmunoprecipitation

Cells (2×10^7) were incubated with or without DEX (10 nM) in the presence or absence of MEL (10 nM) at 37 C. Forty-five minutes after hormone addition, cells were centrifuged for 5 min at 2000 rpm and washed once with cold PBS. Cells were lysed by adding 300 μl of a buffer of 10 mM HEPES (pH 7.5), 1 mM EDTA, 20 mM Na_2MoO_4, 1 mM EGTA, 0.05 mM leupeptin, 1 mM PMSF, and 1 μM pepstatin A and homogenized with a micropotter glass-Teflon. Cell extracts were centrifuged for 10 min at 13,000 rpm at 4 C. Two microliters of BuGR-2 anti-GR antibody were added to 1 mg of proteins. Precipitation was performed by adding 30 μl protein A/G plus agarose solution (Santa Cruz Biotechnology, Santa Cruz, CA; catalog no. sc-2003; 2:1 suspended in buffer of 10 mM HEPES (pH 7.5), 1 mM EDTA, 20 mM Na_2MoO_4, 1 mM EGTA, 0.05 mM leupeptin, 1 mM PMSF, and 1 μM pepstatin A). Samples were mixed by rotation for 2 h at 4 C and centrifuged for 2 min at 13000 rpm. Pellets were washed three times with TEGM buffer of 10 mM HEPES (pH 7.5), 1 mM EDTA, 20 mM Na_2MoO_4, 5% glycerol, and 50 mM NaCl and centrifuged for 2 min at 13000 rpm. Proteins were resolved by Western blotting.

Western blots

Samples from subcellular separation or coimmunoprecipitation assays were electrophoresed for 3 h at 100 V in a 12 or 9%, sodium dodecyl sulfate-polyacrylamide gel, transferred to a polyvinyl difluoride membrane (Bio-Rad Laboratories, Hercules, CA) by electroblotting in transfer buffer containing 20% methanol (vol/vol), 0.19 M glycine, and 0.025 M Tris-base (pH 8.3) at 300 mA for 1.5 h at 0 C. Blots were blocked 1 h at room temperature in Tris-buffered saline [20 mM Tris-Cl (pH 7.5), 500 mM NaCl] containing low-fat powered milk (5%) and Tween 20 (0.1%). The incubations with primary antibodies were performed at 4 C for 12 h in blocking buffer (3% skim milk, 0.1% Tween 20, in Tris-buffered saline) with the following antibody concentrations: 0.02% rabbit polyclonal anti-hsp90 IgG (41) and 0.2% mouse monoclonal Bu-GR2. The membranes were incubated with the corresponding counterantibody (0.03% antirabbit IgG or 0.02% antimouse IgG; Bio-Rad) and the proteins evidenced by enhanced chemiluminescence detection (GE Healthcare, Buckinghamshire, UK). Densitometric analysis of protein levels was performed with ImageQuant software (Molecular Dynamics, Inc., Sunnyvale, CA). The proper loading was evaluated by staining membranes with Ponceau-S.

Cell culture and transient transfection assay

Cells were cultured at 37 C under humidified atmosphere with 5% CO_2 in p100 plates. Cos-7 and L-929 cells were grown in DMEM medium and HC11 cells (kindly provided by Dr. Nancy Hynes, Basel, Switzerland) (42) in RPMI 1640 medium, both supplemented with 10% FCS containing penicillin (100 IU/ml), streptomycin (100 μg/ml), and glutamine (2 mM). For transient transfections, 5×10^5 cells plated in 60-mm plates were transfected with Lipofectin 2000 (Invitrogen) according to the manufacturer's instructions. Briefly, Cos-7 cells were cotransfected with 3 μg MMTV-luciferase vector (43) and 1 μg pRSV-GR (44). pCMV-LacZ (1 μg) was used as a marker for transfection efficiency. Plasmids were diluted in 100 μl medium and added drop-wise to an equal volume of medium containing 2 μl Lipofectin 2000 (Invitrogen) for Cos-7 and HC11 cells, and 4 μl of the reagent for L929 cells. After 20 min, the transfection mixture was added drop-wise to the cells and incubated overnight at 37 C in 5% CO_2 atmosphere. Then the serum-free medium was replaced by regular medium supplemented with 10% charcoal-stripped FCS and antibiotics. Cells were incubated with the corresponding hormones during 48 h for Cos-7 cells and 24 h for HC11 and L929 cells. Luciferase activity was measured with a luciferase kit according to the manufacturer's protocol (catalog no. E1501; Promega Inc., Madison, WI). β-Galactosidase activity was measured as previously described (45).

RNA analysis

RNA was extracted by the single-step method (46). For reverse transcription, 4 μg total RNA were used. The first cDNA strand was synthesized with SuperScript reverse transcriptase (Life technologies) and 25 ng/μl random primers (Invitrogen) as reverse complementary primer. The oligonucleotides 5′-CCGCAACAAGAAGCTCAGGAACTC-3′ (MEL 1A forward) and 5′-TCG TACTTGAGGCTGTGGCAAATG-3′ (MEL 1A reverse) were used as forward and reverse primers, respectively for Mel 1A receptor; the PCR amplified product is 248-bp length (47). For RORα receptor amplification, the oligonucleotides 5′-GCTGACGGAACTGCATGA-3′ (RORa forward) and 5′-TGGATATGTTCTGGGCAAGGT-3′ (RORα reverse) were used as forward and reverse primers, respectively. The PCR product is 280 bp length (48). PCRs were normalized against actin expression. For that, RT-PCRs were performed with the oligonucleotides 5′-TCATGAAGTGTGACGTTGACATCCGT-3′ (ACTIN forward) and 5′-CTTAGAAGCATTTGCGGTGCACGATG-3′ (ACTIN reverse). The correspondent RT-PCR product is 280 bp length (Promega).

The cDNA pool (2 μl), 1.25 U *Thermus aquaticus Taq* polymerase (Invitrogen), and amplification primers (20 pmol each) in 25 μl of PCR mixture (2.5 μl polymerase buffer, 4 mM $MgCl_2$, 250 μM each deoxynucleotide triphosphate) denatured 3 min at 96 C followed by 30 (for actin) or 35 cycles (for Mel 1a and RORα) of amplification by using a step program (1 min at 94 C, 1 min at 60 C, 1 min at 72 C) for Mel-1A; (1 min at 94 C, 1 min at 57 C, 1 min at 72 C) for RORα; (40 sec at 94 C, 30 sec at 70 C, 40 sec at 72 C) for actin; and a final extension at 72 C for 5 min. PCR products were analyzed by electrophoresis in 1.5% agarose gels and visualized under UV light.

Statistical analysis

Results were expressed as means ± SE or means ± SD, as it is indicated in the figure legends. In competition assays, a randomized block design was used to minimize the variability between experiments. Two-way ANOVAs followed by Tukey's multiple comparisons tests were used to detect significant differences among treatments and experiments. In transfection assays, a generalized block design was used also to minimize the variability between experiments, each experiment being considered one level of the experimental factor. Each experiment has two independent replicates per treatment. Two-way ANOVA was also followed by Tukey's multiple comparisons. Statistical analyses were performed with STATISTICA 6.0 (StatSoft, Inc., Tulsa, OK). Differences were regarded as significant at $P < 0.05$. Before statistical analysis data were tested for normality and homoscedasticity using Lilliefors and Bartlett's tests, respectively. The interaction between experimental factor and treatment factor was not significant in all the statistical analysis.

Results

As mentioned before, MEL prevents glucocorticoid-mediated apoptosis in certain cell types (3–7). These results clearly pose an important question: in which step of the molecular mechanism involved in GR action does the methoxyindole impair the ability of activated GR to exert its biological effects?

We first studied whether MEL is able to modulate DEX binding to the GR. Figure 1 shows competition binding assays between DEX and MEL in GR-enriched thymus fractions. MEL did not affect GR binding capacity, suggesting that the methoxyindole should regulate some other downstream step of the GR activation pathway.

Confocal microscopy of immunofluorescence assays demonstrate that inactivated GR was primarily localized in the cytoplasm of thymocytes (Fig. 2, A–D). When cells were incubated with DEX, GR rapidly translocated to the nucleus,

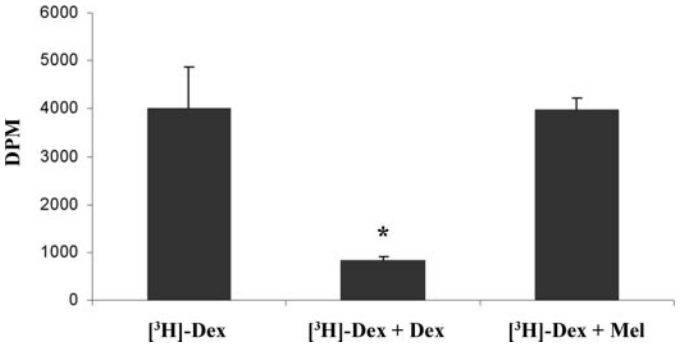

FIG. 1. MEL does not affect binding of DEX to the GR. Competition assay between DEX and MEL for the GR. An enriched thymic proteic fraction of GR was obtained as described in *Materials and Methods*. Six hundred micrograms of proteins were assayed for 16 h at 4 C with ^{3}H DEX ([^{3}H]Dex): 350,000 dpm (Ae = 75 Ci/mmol) in the absence or presence of 5 μM DEX and 5 μM MEL. Each treatment was done in triplicate. Means ± SD from a representative experiment (n = 3) are shown. *, $P < 0.05$ ([^{3}H]Dex + Dex *vs.* [^{3}H]Dex and *vs.* [^{3}H]Dex + Mel).

as confirmed by its colocalization with the specific DNA dye propidium iodide (Fig. 2, E–H). The ability of the ligand to induce the translocation of GR to the nucleus was blocked by MEL (Fig. 2, I–L). MEL did not affect *per se* the subcellular localization of GR (Fig. 2, M–P). This observation was confirmed by subcellular fractionation followed by Western blot assays. Figure 2Q shows that MEL inhibited DEX-mediated GR nuclear translocation. DEX alone promoted GR translocation into the nucleus with respect the untreated control, whereas coincubation with steroid and MEL prevented the GR translocation as shown by similar levels of GR with respect to the control (Fig. 2Q).

To study a possible molecular mechanism for the antagonistic effect of MEL on GR biological effect, we performed immunoprecipitation of GR, obtained from thymocytes in primary culture, followed by Western blots against hsp90. Figure 3 shows that after 45 min of treatment with DEX, hsp90 is almost totally dissociated from GR heterocomplex (Fig. 3, lane 2). Interestingly, the heat shock protein is retained in the complex in the presence of MEL (Fig. 3, lane 3). Incubation with MEL alone does not affect the heterocomplex composition (Fig. 3, lane 4). These results suggest that the methoxyindole prevents GR transformation by blocking the dissociation of hsp90.

To study whether the antagonistic effect of MEL on the GR response is specific of thymocytes or it also occurs in other cell types, a series of studies was performed. A pGR (codifying for human GR) was cotransfected in Cos-7 cells with pMMTV-Luc, which encodes for a luciferase reporter gene

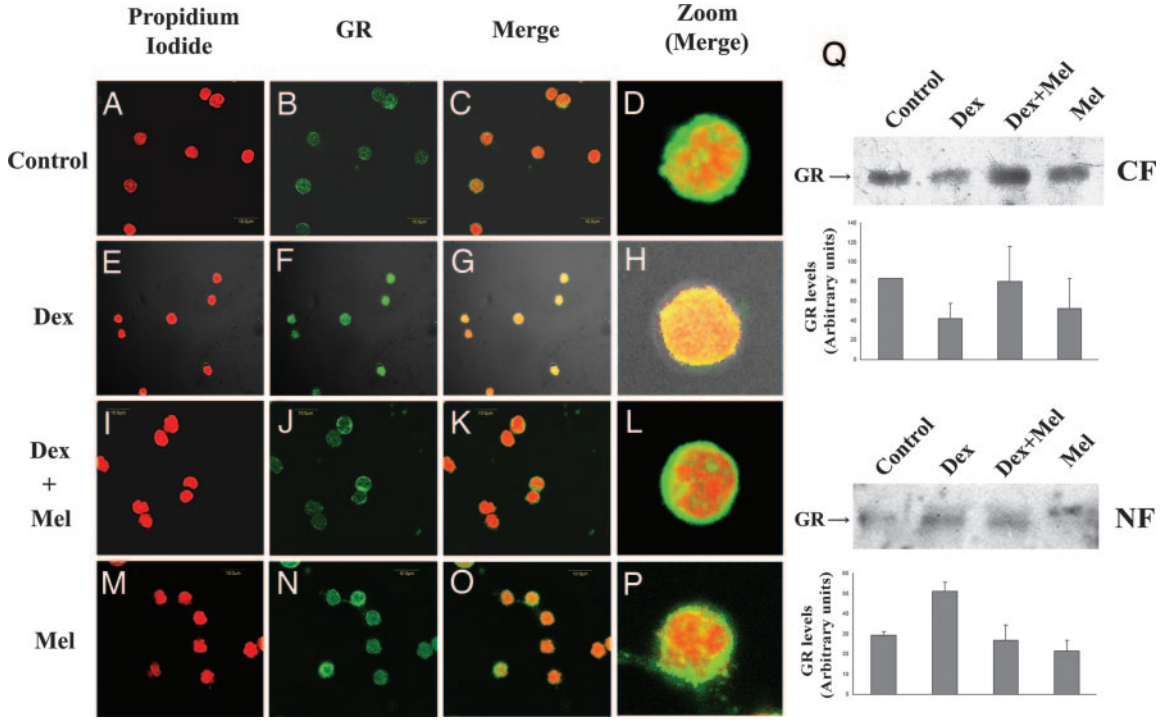

FIG. 2. MEL inhibits nuclear translocation of GR in mouse thymocytes. A–L, Fluorescence confocal microscopy. Primary thymocyte cultured cells were incubated with ethanol (control) (A–D), 10 nM DEX alone (Dex) (E–H), or 10 nM MEL plus 10 nM DEX (Dex + Mel) (I–L) and with 10 nM Mel (M–P) for 30 min at 37 C as described in *Materials and Methods*. Then cells were immunolabeled for GR, treated with propidium iodide to stain cells nucleus, and analyzed by laser fluorescence confocal microscopy as described in *Materials and Methods*. Each field was independently visualized with the appropriate wavelength for propidium iodide (*red*; A, E, I, and M) and GR antibody plus Cy2 dye-conjugated secondary antibody (*green*; B, F, J, and N), and then both images were merged (C, D, G, H, K, L, O, and P). Magnification: ×1000 (A–C, I–K, and M–O); ×800 (E–G); ×4000 (D, H, L, and P). Results are representative of three independent experiments. Q, Subcellular fractionation and Western blot. Cells were incubated for 30 min at 37 C with or without Dex (10 nM) or Mel (10 nM). After incubation, a subcellular fractionation protocol was performed as described in *Materials and Methods* to obtain the nuclear fractions (NF) and cytoplasmatic fractions (CF). Then proteins were processed, and Western blot analysis was performed as described with a mouse monoclonal anti-GR (Q). Densitometric analysis of protein levels was performed with Image Quant software. The proper loading was evaluated by staining the membranes with Ponceau-S. Mean ± SE values of GR levels (in arbitrary units) from three independent experiments are shown.

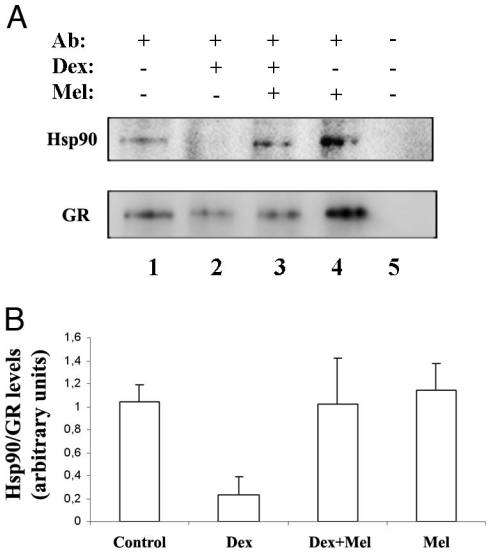

FIG. 3. MEL prevents the dissociation of hsp90 from DEX-induced GR in mouse thymocytes. A, Immunoadsorptions for GR-associated hsp90. Primary thymocyte culture cells were incubated with or without Dex (10 nM) or Mel (10 nM) for 45 min at 37 C. After cells were washed, cytosols were prepared and analyzed for GR-associated hsp90 as described in *Materials and Methods*. Immunoadsorptions were performed with BuGR antibody (Ab) against GR or nonimmune mouse IgG (−). Western blotting was performed with antibodies against GR and hsp90. Gels correspond to one representative experiment. B, Mean ± SE values of hsp90/GR levels (in arbitrary units) from three independent experiments. Densitometric analysis of protein levels was performed with Image Quant software.

under the control of the mouse mammary tumor virus promoter with five GRE elements (43). Figure 4 shows that a 42.2 ± 14.8-fold induction of luciferase activity was observed in cells incubated with DEX (lane 2 *vs.* lane 1). Interestingly, luciferase expression was not significantly different from DEX-treated cells when MEL was coincubated with DEX. (31.9 ± 13.7-fold induction, lane 3), whereas the methoxyindole alone had no effect (1.2 ± 0.1-fold induction, lane 4). Therefore, at least in this cell type, MEL does not affect GR-dependent expression.

As mentioned above, MEL effect could be elicited by its interaction with membrane receptors like Mel 1a, or nuclear receptors such as RORα. According to previous works, Cos-7 cells do not express those receptors (49, 50), so we decided to test luciferase expression in cell types expressing both receptors. Figure 5A shows that both GR-expressing cell lines, HC11 and L929, express both Mel 1a and RORα receptors (Fig. 5A, lanes 3 and 4, respectively), comparable with the expression observed in thymocytes (Fig. 5 A, lane 2).

As Fig. 5, B and C, shows, luciferase activity was induced by DEX in both cell types (4.8 ± 1.2 and 4.4 ± 0.8-fold induction with respect to the control) (Fig. 5, B and C, lane 2 *vs.* lane 1, respectively). Coincubation with MEL has no effect respect to the DEX treatment (4.4 ± 1.0 and 4.3 ± 0.5-fold induction *vs.* control, Fig. 5, B and C, lane 3, respectively). Again, as in Cos-7 treatment, MEL alone was unable to affect luciferase expression (0.8 ± 0.1 and 1.1 ± 0.2-fold induction *vs.* control, Fig. 5, B and C, lane 4, respectively). Therefore, no correlation exists between the presence of melatonin receptors and the ability of MEL to inhibit GR-dependent expression.

To confirm these results, confocal microscopy of immunofluorescence analysis was also performed. Figure 5, D–G, shows that the GR is localized in both the cytoplasm and nucleus of untreated HC11 cells (Fig. 5D). When cells were incubated with DEX, GR is mainly in the nucleus (Fig. 5E), indicating that GR activation is hormone mediated. However, in contrast to those results observed with thymocytes, the coincubation of DEX and MEL did not affect GR nuclear localization induced by DEX (Fig. 5F). Again, MEL alone was unable to affect the subcellular localization of GR (Fig. 5G). Similar results were obtained with L929 fibroblast (data not shown). Taken together, these results suggest a tissue-specific effect of MEL on the hormone-dependent GR activation.

Discussion

Recently our laboratory demonstrated that MEL, an inhibitor of the DEX-mediated apoptosis in thymocytes, inhibits both the glucocorticoid-induced cytochrome C release and the increase in the levels of Bax protein and *bax* mRNA (5). These results are partially in agreement with those previously reported by Sainz *et al.* (11), who showed that preincubation with MEL for 3 h significantly decreases the percentage of DNA fragmentation in thymocytes from 25-d-old rats further incubated for an additional 6-h period with DEX. In our experimental conditions, the antiapoptotic effect of MEL was evident when it was coincubated with DEX for only 3 h without preincubation. However, MEL did not affect the levels of GR under these conditions (5). The antagonistic effect between MEL and glucocorticoids was also reported by Kiefer *et al.* (51) in MCF-7 cells. These authors (51) showed that the methoxyindole can significantly diminish DEX-induced GR transcriptional activation.

The mechanism of action of the GR involves a direct interaction of a glucocorticoid ligand with the receptor. Once

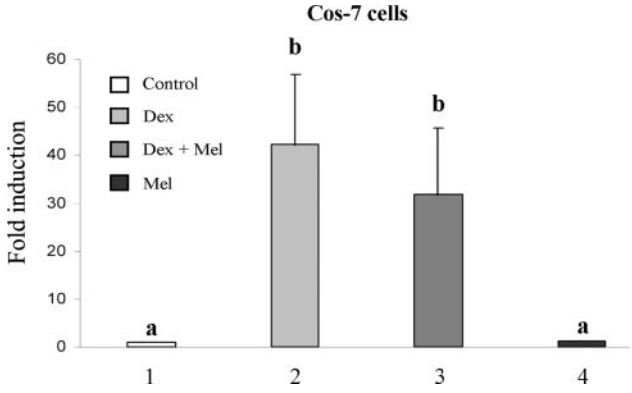

FIG. 4. MEL does not affect transcriptional activity of DEX-activated GR in Cos-7 cells. Transcriptional activity assay of GR. COS-7 cells were cotransfected with 1 μg pRSV-GR and 3 μg MMTV-Luc reporter vector. One microgram of pCMV-LacZ vector were also introduced. Cells were incubated for 48 h with ethanol (control; lane 1), 10 nM DEX (lane 2), 10 nM DEX plus 10 nM Mel (lane 3), or 10 nM Mel (lane 4), and luciferase activity was measured. After correcting for β-galactosidase activity, values were expressed as fold induction relative to the controls. Means ± SE from three independent experiments are shown. *Bars with different superscript letters* are significantly different from each other ($P < 0.05$).

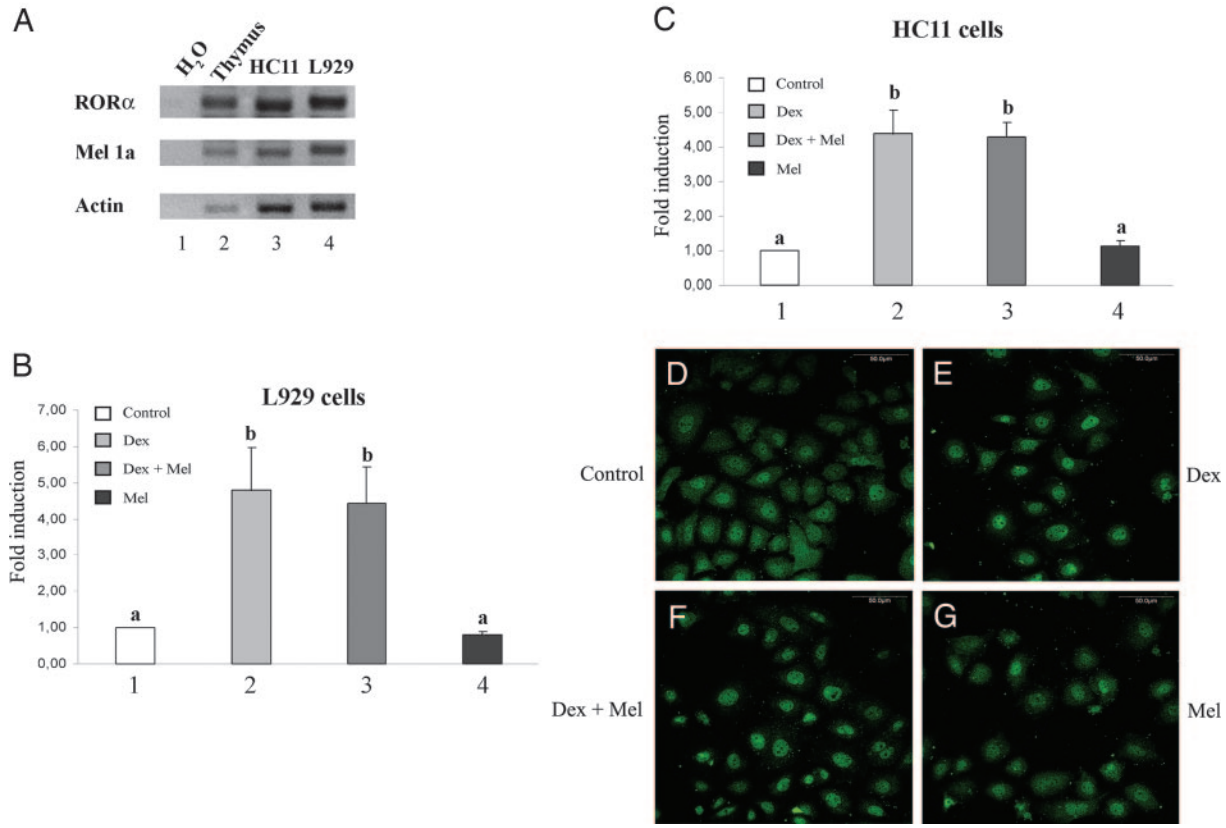

FIG. 5. MEL affects neither transcriptional activity nor nuclear translocation of DEX-activated GR in cells expressing both Mel 1a and RORα receptors. A, Expression of Mel-1a and RORα receptors. Total RNA from thymocytes, HC11, or L929 cells was extracted and RT-PCR with specific primers for Mel-1a, RORα, and actin were performed as described in *Materials and Methods*. Lane 1, Negative control. B and C, Transcriptional activity assay of GR. L-929 cells (B) or HC11 cells (C) were transfected with 3 μg of MMTV-Luc reporter vector. One microgram of pCMV-LacZ vector were also introduced. Cells were incubated for 24 h with ethanol (control; lane 1), 10 nM DEX (lane 2), 10 nM DEX plus 10 nM Mel (lane 3), or 10 nM Mel (lane 4), and luciferase activity was measured. After correcting for β-galactosidase activity, values were expressed as fold induction relative to controls. Means ± SE from three independent experiments are shown. *Bars with different superscript letters* are significantly different from each other ($P < 0.05$). D–G, Fluorescence confocal microscopy. HC11 cells were incubated for 30 min with control (D), Dex (10 nM) (E), Dex + Mel (10 nM) (F), or Mel (10 nM) (G). Then cells were immunolabeled for GR and analyzed by laser fluorescence confocal microscopy as described in *Materials and Methods*. Magnification: ×400. *Bar*, 50 μm. Results are representative of three independent experiments.

the ligand/GR complex is formed, the precise conformation adopted by the receptor is determined by the structure of a given ligand (for a review see Ref. 52).

In this paper, we showed that in cytosolic fractions of thymocytes, MEL is not able to compete with DEX for the binding to the GR. However, we can neither dismiss the possibility that the methoxyindole may compete with glucocorticoids *in vivo* nor that MEL binds to the GR in a second binding site different from the glucocorticoid binding pocket. But if a second binding site would exist, MEL binding would not affect the ability of the receptor to bind DEX. In this sense, it has been suggested that the synthetic agonist 11,19-oxidoprogesterone binds to a second site in a closely related member of the steroid receptor superfamily, the mineralocorticoid receptor (53). The biological effect of that steroid seems to be mineralocorticoid receptor mediated through a putative regulatory binding site that could be different from the classical aldosterone-binding pocket (53). However, it seems unlikely that a similar mechanism could take place with the glucocorticoid receptor because the antagonistic effect of MEL is not observed in other experimental models such as Cos-7, HC11, and L929 cell lines, suggesting an indirect and thymocyte-specific action of the methoxyindole. Thus, MEL would inhibit GR activity through the activation of specific signal transduction pathways.

Several works have shown that the inhibitory effect of MEL on GR activation involves the inhibition of GR interaction with specific GREs that are present in its target promoters. However, those reports do not precisely account which step between the ligand/GR complex formation and the receptor/DNA interaction is affected by MEL. Actually, most of those studies were limited to *in vitro* gel-shift assays with nuclear extracts glucocorticoids-treated cells (30, 51, 54). In this paper, we show that MEL does antagonize the glucocorticoid response by preventing GR dissociation from the hsp90-based heterocomplex and retaining the GR in the cytoplasm. At this point, it is still uncertain whether the tight MEL-induced association of hsp90 to the GR is directly responsible for the cytoplasmic retention of the receptor or whether MEL provokes the retention through an indirect mechanism. Nonetheless, the final effect evoked by MEL is the inhibition of the GR transformation. In this sense, it is interesting to point out that the hsp90-immunophilin complex associated to GR seems to be related to the transport of

the receptor throughout the cytoplasm (55). When the heterocomplex is disrupted, the GR nuclear translocation is impaired, even though the chaperone complex is still associated with the receptor (31). Therefore, it might be possible that MEL plays a similar role in preventing the retrograde transport of the GR.

The inhibition of GR nuclear translocation by the activation of a given pathway was also suggested by Goleva *et al.* (56), who demonstrated that a GR signal transducer and activator of transcription-5 heterodimer prevents the nuclear import of GR, suggesting a novel role for signal transducer and activator of transcription-5 in IL-2-induced steroid insensitivity (56).

Because the inhibitory effect of MEL was not observed in other cell types such as HC11 and L929 cells, it is possible to speculate that MEL could not bind directly to any of the proteins of the GR/hsp 90 heterocomplex, but it activates some tissue-specific pathway that leads to GR nuclear translocation blockage; for example, MEL might affect the GR phosphorylation state, which plays a key role in GR subcellular destination (57). Recently it was demonstrated in human T cells that phosphorylation of GR by ERK1/2 inhibits DEX-mediated GR nuclear localization (58).

On the other hand, it was also demonstrated that MEL antagonize the Ca^{2+} binding protein calmodulin (CaM) through a direct interaction of the methoxyindole with the CaM α-subunit (59, 60). As it was suggested, CaM could be involved in the modulation of GR functions through its interaction with the GR-associated hsp90 (59, 61), allowing the speculation that CaM would participate in the MEL/GR antagonism. However, this putative mechanism, involving a direct interaction Mel/CaM, would not explain the tissue-specific effects that we describe here.

In the thymus, two types of MEL receptors are expressed. Mel 1 membrane receptors that have been described in rat T and B lymphocytes (18) and the nuclear receptors RZR/RORα, RORβ, and RZRγ (18, 20).

The activation of Mel 1a by MEL decreases cAMP accumulation in mouse thymocytes (19). However, because DEX also decreases cAMP levels in thymocytes, this pathway does not seem to be involved in the inhibitory effect exerted by MEL (5). In addition, activation of Mel 1a also regulates the level of other second messengers such as cGMP (19, 62) and cytosolic Ca^{2+} (16). Thus, we cannot discard the possibility that some of them could be involved in the effect of MEL.

On the other hand, as mentioned above, MEL binds to purified thymocyte nuclei (29), suggesting the existence of functional nuclear receptors of the ROR family. In other tissues MEL regulates the expression of several genes through that receptor (27, 28). Therefore, the RORα/MEL complex could be also a possible candidate to account for the inhibition of the DEX-GR complex translocation to the nucleus. In addition, a relationship between GR and RORα has been suggested because the GR-interacting protein-1 functions as a coactivator for the RORα receptor (63).

Thus, we speculate which signaling pathway would mediate the inhibitory effect between MEL and GR. In this sense, several reports pointed out the membrane receptor Mel 1a as a putative candidate (30). In fact, it was demonstrated in MCF7 cells that the action of MEL seems to be sensitive to pertussis toxin, indicating a possible involvement of Gi protein coupled to Mel 1a (51).

Our results show that despite both receptors, Mel1a and RORα, are expressed in HC11 and L929 cell lines, melatonin is unable to antagonize GR nuclear translocation. Furthermore, this absence of correlation between the presence of melatonin receptors and the ability of MEL to inhibit GR-dependent expression suggests that the tissue specificity for this effect would be on one hand, independent of the melatonin receptors or, on the other hand, owing to tissue specific factors acting downstream the receptors, in the signal transduction pathway.

In summary, the present work shows for the first time the molecular mechanism involving the GR/MEL antagonism by which this methoxyindole inhibits GR nuclear translocation and hsp90 dissociation. The apparent tissue specificity for this mechanism is a matter of further studies.

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