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Melatonin inhibits glucocorticoid-dependent GR–TIF2 interaction in newborn hamster kidney (BHK) cells

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ABSTRACT

The antagonism exerted by melatonin on the glucocorticoid response has been well established, being strongly dependent on the cellular context. Previously, we found that melatonin inhibits glucocorticoid receptor (GR) dissociation from the chaperone hetero-complex and nuclear translocation on mouse thymocytes. Here, by performing confocal fluorescence microscopy and the *Number and Brightness* assay we show that in newborn hamster kidney cells (BHK21) melatonin neither affects GR nuclear translocation nor GR homodimerization. Instead, co-immunoprecipitation studies suggest that physiological concentrations of melatonin impair GR interaction with the transcriptional intermediary factor 2 (TIF2). This melatonin effect was not blocked by the MT_1/MT_2 receptor antagonist luzindole. Curiously, luzindole behaved as an antiglucocorticoid *per se* by impairing the glucocorticoid-dependent MMTV-driven gene expression affecting neither GR translocation nor GR–TIF2 interaction.

1. Introduction

Melatonin (*N*-acetyl-5-methoxytryptamine) is known to be involved in several physiological functions, including the entrainment of seasonal and circadian rhythms. In humans, melatonin participates in the regulation of sleep, seasonal disorders and aging. Moreover, antitumoral properties of this hormone, as well as its involvement in the responsiveness of the immune system have been described. Lastly, melatonin and its metabolic derivates function as a broad-spectrum antioxidant and a free radical scavenger (Reiter et al., 2010).

Some of melatonin's effects are attributed to its interaction with the membrane receptors MT_1 and MT_2, acting mainly through a cAMP-dependent pathway (Vanecek, 1998). On the other hand, numerous data support the fact that this indoleamine readily passes through cell membranes and seemingly has access to all sub-cellular organelles (Hevia et al., 2008; Reiter et al., 2010). In this context, experimental data support the idea that the antioxidant melatonin protects tissues against free radicals-mediated damage (Laothong et al., 2010; Milczarek et al., 2010). Furthermore, it was suggested that melatonin modulates the activity of Ca^{2+} binding proteins such as calmodulin (del Rio et al., 2004).

Glucocorticoids (GCs) are involved in numerous physiological processes such as cellular proliferation, differentiation, apoptosis and metabolism (Franchimont, 2004), forming also the basis of current anti-inflammatory and immunosuppressive therapies. GR localizes in the cytoplasm bound to a multiprotein heterocomplex composed by chaperones, including hsp90, hsp70, and immunophilins among others (Pratt et al., 2004). GCs can freely diffuse across the plasma membrane and once in the cytoplasm, they interact with GR, leading to its activation. Activated GR translocates into the nucleus, dissociates from chaperone complex and regulates gene expression (Necela and Cidlowski, 2004). The classical mode of action involves GR binding to glucocorticoid response elements (GRE) located in promoter region of target genes. For transcription to occur the subsequent recruitment of co-activator proteins is also required (Xu and Li, 2003). GR interacts with the p160 family of coactivators, which includes SRC-1, SRC-2 (also known as TIF2) and SRC-3 (McKenna et al., 1999). On the other hand, GR is capable of modulating gene expression by an indirect mechanism where the receptor interacts with other transcription factors, such as NFκB, AP-1 or STATs, by regulating their transcriptional activities (Kassel and Herrlich, 2007).

Several reports propose a link between melatonin and glucocorticoids activities. In fact, it has been suggested that melatonin

Abbreviations: BHK21, baby hamster kidney cells; DEX, dexamethasone; EGFPGR, enhance green fluorescence protein; EMSA, electro mobility shift assay; GCs, glucocorticoids; GR, glucocorticoid receptor; GRE, glucocorticoid response elements; LUZ, luzindole; MEL, melatonin; MMTV, mouse mammary tumor virus; N&B, number and brightness; TIF2, transcriptional intermediary factor 2.

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modulation of the immune system is carried out through its inhibitory effect on glucocorticoid actions (Nelson and Drazen, 1999; Provinciali et al., 1996; Sainz et al., 1995). Though the molecular mechanism regarding the antagonism between melatonin and GCs are still unclear, it was shown that melatonin inhibits GR mRNA expression (Sainz et al., 1999) and modulates GR ligand interaction (Blackhurst et al., 2001). In a previous work, we have demonstrated that melatonin inhibits apoptosis by preventing cytochrome C release and reducing glucocorticoid mediated-increase in Bax levels (Hoijman et al., 2004). Melatonin also prevents dexamethasone-induced gene expression (Persengiev, 1999); suggesting that the methoxyindole may control the ability of activated GR to reach their specific target genes. In this sense, we have found that melatonin impairs GR nuclear translocation on murine thymocytes, most likely by blocking GR-Hsp90 dissociation (Presman et al., 2006).

In the present work, we present a novel mechanism by which melatonin antagonizes glucocorticoid action. In the kidney-derived hamster BHK21 cells, we found that melatonin does not inhibit GR nuclear translocation nor GR homodimerization, but rather reduces GR interaction with TIF2 co-activator, impairing in this way GR mediated induction of MMTV-driven luciferase expression. Surprisingly, we also found that the well-known MT_1/MT_2 receptor antagonist luzindole have *per se* potentially antiglucocorticoid properties; as it is capable of inhibiting glucocorticoid-dependent gene expression on BHK21 and Cos-7 cells.

2. Material and methods

2.1. Reagents and hormones

Dexamethasone (DEX), melatonin (MEL), luzindole (LUZ) and 2′0-monosuccinyladenosine-30,50-cyclic monophosphate tyrosyl methyl ester (TME-cAMP) was purchased from Sigma–Aldrich (St. Louis, MO, USA); Dulbecco's modified Eagle's medium (DMEM) from Invitrogen (Carlsbad, CA, USA) and Fetal calf serum (FCS) from Internegocios S.A. (Buenos Aires, Argentina). Charcoal-stripped FCS was prepared with charcoal-dextran as described elsewhere (Lippman et al., 1976). DEX and melatonin were prepared as 1000X stock solution in ethanol and used at a final concentration of 10 nM and 100 nM, respectively. Luzindole was prepared as stock solutions (10 mM in DMSO) and stored in small aliquots at −20 °C. The TME-cAMP was radiolabeled with $Na^{125}I$ in our laboratory by the method of chloramine T (specific activity, 600Ci/mmol) previously described (Del Punta et al., 1996). The specific antibody for cAMP was provided by Dr. A.F. Parlow (National Hormone and Pituitary Program of the NIDDK).

2.2. Cell culture and transient transfection assays

BHK21 and Cos-7 cells were cultured in DMEM supplemented with 10% FCS plus penicillin (100 IU/ml) and streptomycin (100 μg/ml) at 37 °C under humidified atmosphere with 4.5% CO_2. Transient transfections were performed with Lipofectin 2000 (Invitrogen) according to manufacturer's instructions. Briefly, 3×10^4 BHK21 cells were co-transfected on 24-well plates with 0.3 μg pMMTV-luciferase (Horwitz et al., 1978) vector and/or 0.3 μg pEGFPGR (Galigniana et al., 1998) when indicated; 0.2 μg pCMV-LacZ was added as transfection control in transactivation assays. Cos-7 cells (3×10^5) were co-transfected in 60 mm plates with 3 μg pMMTV-luciferase or 3 μg BclX-P4-Luciferase (Viegas et al., 2004), 1.5 μg pCMV-LacZ and 1 μg pRSV-hGR (Godowski et al., 1987) as previously described (Presman et al., 2006). After transfection, cells were incubated in DMEM containing 5% charcoal-stripped FCS and the corresponding compounds from 1000-fold stock solutions as indicated in each experiment. Control samples were treated with vehicle (Ethanol and/or DMSO). Luciferase activity was measured according to the manufacturer's protocol (Promega Inc. cat # E1501). β-galactosidase activity was measured as previously described (Truss et al., 1995).

2.3. Cellular localization, intranuclear distribution and N&B analysis

Cells (3×10^5) were transfected with 1 μg of pEGFPGR (Galigniana et al., 1998) or the empty vector pEGFP-C3 (Clontech) and incubated with the corresponding hormones for at least 40 min. Next, the medium was replaced with RAB buffer (Hepes 10 mM pH 7.4; NaCl 135 mM, KCl 10 mM, $MgCl_2$ 0.4 mM; $CaCl_2$ 1 mM; Glucose 1%) supplemented with the indicated hormones and then analyzed by confocal fluorescence microscopy. All measurements were done in a FV1000 confocal laser scanning microscope (Olympus), with an Olympus UPlanSApo 60X oil immersion objective (NA = 1.35). The excitation source was a multi-line Ar laser tuned at 488 nm (average power at the sample, 700 nW). Fluorescence was detected with a photomultiplier set in the pseudo photon-counting detection mode. Intranuclear analysis and *N&B* measurements were done as previously described (Presman et al., 2010).

2.4. Electro mobility shift assay (EMSA)

EMSAs were performed as previously described (Presman et al., 2010). For binding studies, reactions were carried out in 25 μl reaction buffer (5 mM Tris–HCl, pH 8, 0.5 mM EDTA, 5% glycerol, 0.5 mM 2-mercaptoethanol, 1 μg poly(dI–dC), 90 ng Calf thymus DNA, and 30 μg BSA), 10 ng of radiolabeled probe and 200-fold excess of unlabeled specific or unspecific probe when indicated. Three micrograms of the nuclear extract were added to the binding reaction and incubated for 20 min at room temperature. Then, reaction mixtures were subjected to 6% acrylamide gel in Tris-Borate-EDTA solution. For dissociation studies, reactions were carried out in 100 μl reaction buffer containing 50 ng of radiolabeled probe and 18 μg of nuclear extract. After 20 min incubation at room temperature, 200-fold unlabeled probe was added and reaction aliquots (16 μl) were loaded at different times into a gel running at 200 V. Images were taken with STORM 820 PhosphorImager and analyzed with NIH-Image J v1.63 software analysis. For the comparison of DNA dissociation, all extracts showed similar DNA binding at the initial time point prior to the addition of excess GRE; thus, dissociation rates were independent of the fraction of DNA bound.

2.5. Co-immunoprecipitation and western blot assays

BHK21 cells (2×10^6) co-transfected on 100 mm-plates with 5 μg pRSV-hGR (Presman et al., 2010) and 5 μg pTIF2 (Nojek et al., 2004) were incubated with the corresponding hormones for 90 min at 37 °C. Then, cells were lysated with CytoBuster Protein Extraction Buffer (EMD Biosciences, Darmstadt, Germany) supplemented with Protease inhibitor cocktail (Calbiochem, San Diego, CA, USA) and clarified by centrifugation at 15,000*g* for 5 min at 4 °C. Protein extracts (1 mg per treatment) were precleared with protein A/G plus agarose solution (Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-2003). Samples were mixed by rotation for 1 h at 4 °C and centrifuged for 2 min at 15,000*g*. Supernatants were immunoprecipitated with TIF2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-8996) and protein A/G plus agarose solution. Samples were then mixed by rotation for 4 h at 4 °C and centrifuged for 3 min at 15,000*g*. Pellets were washed three times with TEGM buffer (10 mM HEPES pH 7.5, 1 mM EDTA, 20 mM Na_2MoO_4, 5% glycerol, 50 mM NaCl) and centrifuged for 3 min at 15,000*g*. Proteins were extracted with SDS sample buffer,

separated in 7% SDS–PAGE and transferred to PVDF membrane (Bio-Rad) by electroblotting. Immunodetection was achieved with a 1:1 mixture of GR antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-1002; sc-1003).

2.6. cAMP assessment

Intracellular cAMP was determined as described previously (Mondillo et al., 2009). After a 30-min incubation of the BHK21 cells with any combination of drugs in the presence of 0.5 mM IBMX, the plates were placed on ice and the medium was aspirated. Next, 0.5 ml of cold distilled water was added to each well, and cells were scraped and disrupted by ultrasonic oscillation. Samples were heated in boiling water three times during 1 min to destroy endogenous protein kinase. After centrifugation for 3 min, samples were diluted using 50 mM sodium acetate buffer (pH 6.0). Unknown samples and standards were acetylated and assayed by RIA. The inter-assay and intra-assay variations of coefficients were lower than 10%.

2.7. Statistical analysis

Results were expressed as means ± standard error measure (SEM). Statistical analyses were performed with STATISTICA 7.0 (StatSoft, Inc.) and consisted of one- or two-way ANOVA followed by Tukey's multiple comparisons tests. Transient transfection experiments have three independent replicates per treatment while RIA measurements have two independent replicates per treatment. 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. In all cases, bars with different superscript letters are significantly different from each other.

3. Results

3.1. Melatonin inhibits GR–TIF2 interaction in BHK21 cells

Melatonin ability to antagonize GCs depends on cellular context. While melatonin inhibits glucocorticoid action in a few cell types, including thymocytes and neuronal cells (Hoijman et al., 2004; Quiros et al., 2008; Sainz et al., 1995); other cell lines such as Cos-7, L929, or HC11 cells are resistant to melatonin effect on glucocorticoids (Presman et al., 2006). Melatonin inhibition of DEX ability to induce MMTV-driven luciferase expression was also observed by transient transfection assays on the newborn hamster kidney-derived BHK21 cell line (Fig. 1). This result adds another cell type to the list of experimental models in which melatonin antagonizes GCs effects.

We wondered whether melatonin affects GR sub-cellular distribution in BHK21 cells as occurs in thymocytes and neuronal cells. Thus, a GFPGR vector was overexpressed in this cell line and its distribution upon hormone-treatment was evaluated by confocal microscopy. Fig. 2A shows that most of the fluorescence localizes into the cytoplasm in untreated cells (control) or in cells treated with melatonin alone. However, as expected, upon DEX addition GFPGR fluorescence localizes almost completely into the nuclear compartment. Interestingly, melatonin did not prevent GFPGR translocation (Fig. 2A) despite its inhibition of DEX-dependent GFPGR-mediated MMTV-activation (Fig. 2B). This result suggests

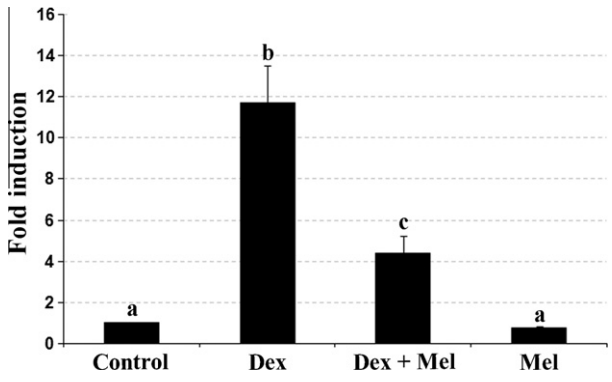

Fig. 1. DEX-induced GR transcriptional activity is inhibited by melatonin in BHK21 cells. BHK21 cells were transfected with pMMTV-Luciferase reporter and pCMV-LacZ vectors. Cells were incubated for 18 h with ethanol (Control), 10 nM DEX, 10 nM DEX and/or 100 nM MEL as indicated. Values were expressed as fold induction (luciferase/β-galactosidase activity) relative to the control. Means ± S.E. from four independent experiments are shown. Bars with different superscript letters are significantly different from each other ($P < 0.05$).

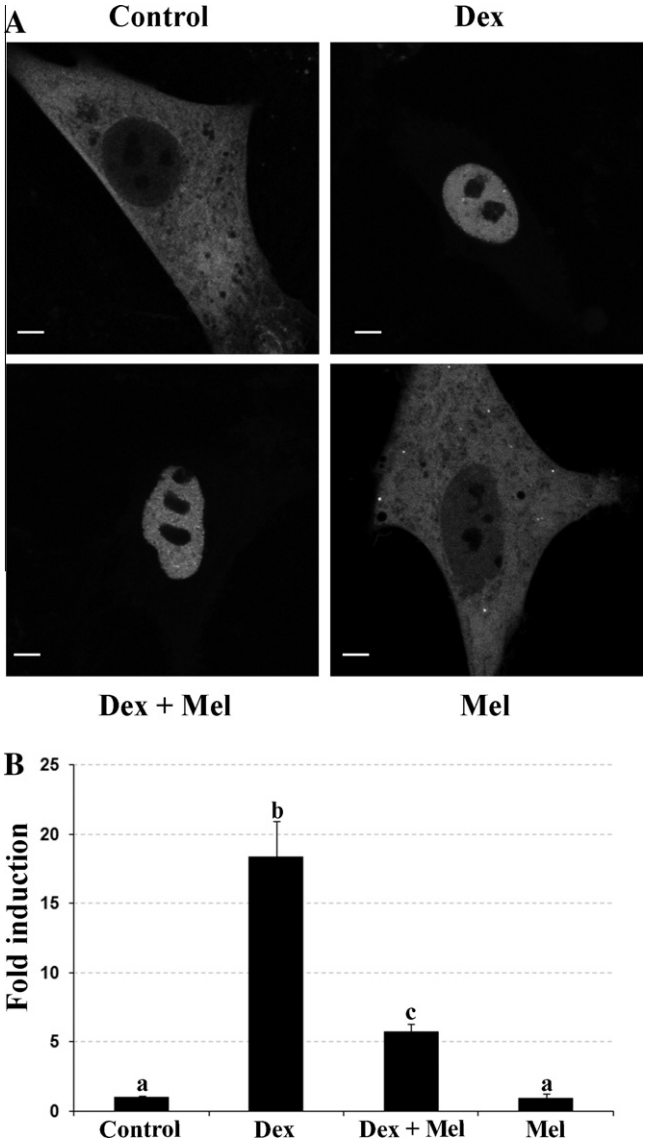

Fig. 2. GFPGR nuclear translocation is not affected by melatonin. BHK21 cells transfected with pEGFPGR (A) or pMMTV-Luciferase plus pCMV-LacZ plus pEGFPGR (B) and incubated with 10 nM DEX and/or 100 nM MEL as indicated. (A) Cells were visualized by confocal scanning microscopy. The figure shows one representative cell for each treatment. Scale bar = 20 μm. (B) Values were expressed as fold induction (luciferase/β-galactosidase activity) relative to the control. Means ± S.E. from four independent experiments are shown. Bars with different superscript letters are significantly different from each other ($P < 0.05$).

that in BHK21 cells, melatonin modulates GR activity through a different mechanism from that observed in thymocytes (Presman et al., 2006) or neuronal cells (Quiros et al., 2008), by affecting an event downstream GR nuclear translocation.

Previous published studies have shown that active GR–ligand complexes form focal domains into the nucleus, consisting of several receptor molecules (van Steensel et al., 1995). However, while some authors found a correlation between ligand dependent GR intranuclear distribution and transcriptional activity (Htun et al., 1996), other ones suggest that the foci distribution would depend on affinity-based differences between ligands rather than on GR transcriptional properties (Presman et al., 2010; van Steensel et al., 1995). Accordingly, GR foci formation in the presence of MEL was then evaluated. Fig. 3A shows that MEL does not affect GFPGR intranuclear distribution triggered by DEX-treatment.

GR homodimerization is considered an essential step in the receptor's transactivation pathway (Necela and Cidlowski, 2004), therefore we wondered whether melatonin prevents GR activation by inhibiting GR dimer formation. To test this hypothesis, *in vivo* mapping of GR oligomerization state was performed by using the *Number and Brightness* (*N&B*) assay (Presman et al., 2010). This new technique, based on moment-analysis, provides the average number of moving, fluorescent molecules and their brightness at every pixel of images (Digman et al., 2008). Fig. 3B shows the real

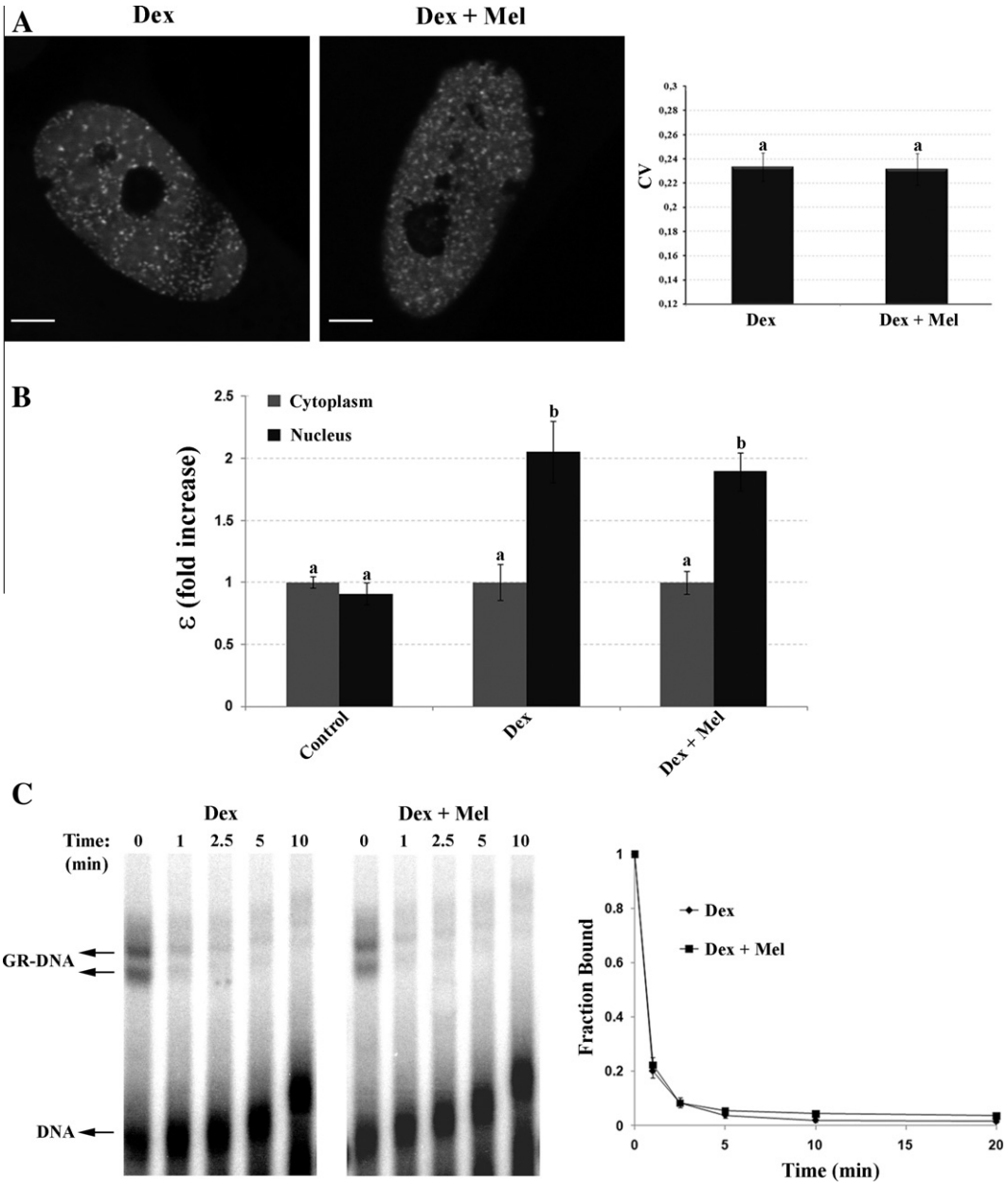

Fig. 3. GFPGR intranuclear distribution, receptor homodimerization and GR–DNA *in vitro* dynamics are not affected by melatonin. BHK21 cells transfected with pEGFPGR were incubated with 10 nM DEX and/or 100 nM MEL as indicated. (A) Cells were visualized by confocal scanning microscopy. The figure shows representative cells for each treatment and the coefficient of variation (CV) quantitation as previously described (Presman et al., 2010). Scale bar = 20 μm. (B) Images were taken between 40 min and 6 h after hormone addition. Previous studies indicated that the intensity of the signals for nuclear and cytoplasmic GR appear to be stable over that time-frame (Presman et al., 2010). For each cell ($15 \leqslant n \leqslant 30$ per treatment) the apparent brightness was calculated as previously described (Presman et al., 2010). The figure shows fold-increase of the real brightness (ε) relative to the cytoplasm for each cell type. Bars with different superscript letters are significantly different from each other ($P < 0.05$). C. Nuclear protein extracts were prepared and incubated with a ^{32}P-radiolabeled oligonucleotide containing a *GRE* sequence. Aliquots for each treatment were loaded into a running gel at the indicated times and images were analyzed. The figure shows a representative gel and the dissociation curves (mean ± S.E.) from three independent experiments showing the fraction bound (relative to time zero) expressed as the ratio between GR–DNA complexes and free DNA probe. The *arrows* indicate the free DNA and the GR–DNA complexes.

brightness (ε) fold increase (a measure of fluorophore oligomerization) corresponding to GFPGR protein expressed in BHK21 cells. In the absence of ligand, ε values are similar in nucleus and cytoplasm; indicating the same oligomerization status of GR in both cellular compartments. However, upon DEX addition ε values significantly increased (approximately 2 fold) in the nucleus with respect to the cytoplasm, consistent with GR dimerization. Interestingly, in the presence melatonin ε values are similar to those observed with DEX alone. Therefore, we conclude that melatonin does not affect GR dimer formation.

In order to test whether melatonin modulates GR–DNA interaction, we performed gel-shift assays with nuclear extracts obtained from BHK21 cells treated with Dex in the presence or absence of melatonin. Extracts were incubated with a radiolabeled oligonucleotide containing two GRE sites from the MMTV promoter. As it was previously reported, nuclear extracts prepared in the absence of ligand generate a double retarded band corresponding to GR–MMTV probe complexes which does not differ from ligand treated extracts (Pandit et al., 2002). Accordingly, melatonin does not affect GR–DNA association (Fig. 3C; compare time 0 min, Dex vs. Dex + Mel). Given this lack of ligand effects on the GR affinity for GRE-sites, we investigated whether melatonin-treatment affects the association/dissociation rate between Dex-GR complexes and DNA. Results show that melatonin had no appreciable effect on GR–DNA interaction dynamics (Fig. 3C).

GR ability to induce gene expression depends on the receptor's capacity to recruit co-activators (Collingwood et al., 1999). There-

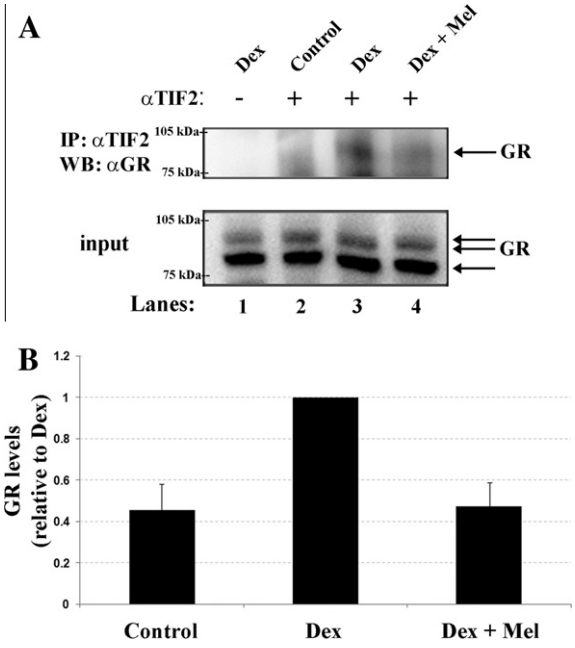

Fig. 4. Melatonin prevents GR–TIF2 association in BHK21 cells. BHK21 cells co-transfected with pRSV-hGR and pTIF2 vectors were incubated with the indicated hormones for 90 min at 37 °C. Cells were washed; cytosols were extracted and analyzed for TIF2-associated GR as described in "Materials and Methods". Immunoadsorptions were performed with TIF2 antibody (αTIF2). Inputs correspond to 10% of the sample previously to the immunoprecipitation protocol. Arrows on input gel indicate the three isoforms of αGR (GR_A; GR_B; GR_C as previously described (Presman et al., 2010)). Gels in A correspond to one representative experiment ($n = 3$). Mean ± SE values of immunoprecipitated (IP) GR levels (respect to Dex treatment) are shown (B).

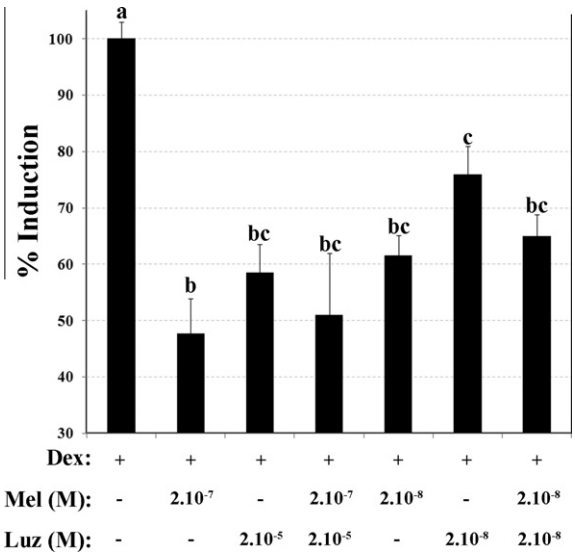

Fig. 5. Luzindole effect on MMTV-driven gene expression. BHK21 cells were transfected with pMMTV-Luciferase reporter vector. Cells were incubated for 18 h with a combination of 10 nM DEX and the indicated concentrations of MEL and/or LUZ. Values were expressed as% induction (luciferase/β-galactosidase activity) relative to DEX treatment. Means ± S.E. from five independent experiments are shown. Bars with different superscript letters are significantly different from each other ($P < 0.05$).

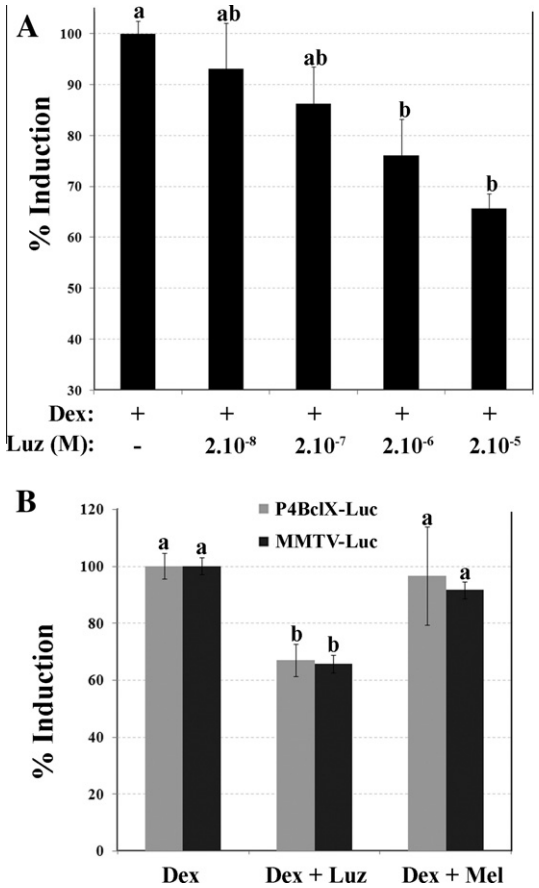

Fig. 6. Luzindole as a potential antiglucocorticoid. (A) Cos-7 cells were transfected with pRSV-hGR and pMMTV-Luciferase reporter vector. Cells were incubated for 18 h with increasing concentrations of luzindole (Luz) in the presence or absence of 10 nM DEX. (B) Cos-7 cells were transfected with pRSV-hGR and pMMTV-Luciferase reporter vector or BclX.P4-Luciferase reporter vector. Cells were incubated for 18 h with 10 nM DEX, 20 μM LUZ (MMTV-Luc), 20 nM Luz (BclX.P4-Luc) or 100 nM MEL. Values were expressed as% induction (luciferase/β-galactosidase activity) relative to DEX treatment. Means ± S.E. from at least four independent experiments are shown. Bars with different superscript letters are significantly different from each other ($P < 0.05$).

fore, one might postulate that melatonin could antagonize GR activation through the impairment of co-activators recruitment. To test this hypothesis, GR–TIF2 co-immunoprecipitation (Co-IP) assays were performed with BHK21 cells co-transfected with hGR and TIF2 expression vectors. Cells were treated with DEX in the presence or absence of melatonin. Subsequently, protein extracts were precipitated with a specific TIF2 antibody and western blot analysis were performed against GR. Results showed that GR is not able to co-precipitate with the coactivator in the presence of melatonin (Fig. 4).

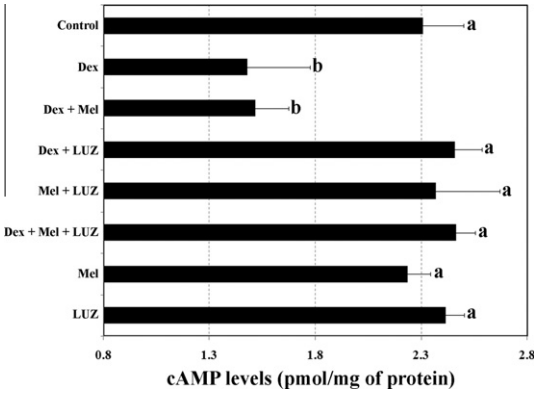

Fig. 7. Dexamethasone, melatonin and luzindole effect on intracellular cAMP levels in BHK21 cells. BHK21 cells were treated for 30 min with vehicle (Control); 10 nM DEX, 100 nM MEL, 20 μM LUZ or any combination indicated. Then, a *RIA protocol* was performed as described in "Material and Methods" section. The figure shows Means ± S.E intracellular cAMP levels expressed as [pmol/mg of protein extract]; from two independent experiments performed by duplicate. Bars with different superscript letters are significantly different from each other ($P < 0.05$).

3.2. Luzindole as a potential antiglucocorticoid

As we mentioned above, most of melatonin's effects have been attributed to its interaction with the membrane receptors MT_1 and MT_2. Thus, we asked whether these receptors are involved in the signaling pathway involved on melatonin/GCs antagonism. Since luzindole has been characterized as a specific MT_1 and MT_2 antagonist (Boutin et al., 2005), melatonin's ability to inhibit GR-dependent gene expression in the presence of this inhibitor was tested. Surprisingly, luzindole inhibited GR transcriptional activity, independent of the presence of melatonin (Fig. 5). Moreover, no additive effect was observed when both methoxyindoles where added, either in high or low doses. This *per se* action of luzindole does not allow us to evaluate the potential of MT receptors to be involved on the melatonin signaling pathway.

In order to test the possible antiglucocorticoid activity of luzindole, we analyze glucocorticoid activity on Cos-7 cells expressing the hGR protein. This cell line does not express MT receptors (Roca et al., 1996). Interestingly, a dose-dependent antiglucocorticoid effect of luzindole was observed in this cell line (Fig. 6A), where melatonin is unable to block GR action (Fig. 6B and (Presman et al., 2006)). Consistently, luzindole also inhibited the activation of the murine bcl-X.P4 promoter (Fig. 6B), which is also responsive to glucocorticoids (Viegas et al., 2004). This result indicates that the antiglucocorticoid activity of luzindole is not only restricted to the MMTV promoter.

Since MT_1 and MT_2 receptors acts mainly through a cAMP-dependent pathway (Vanecek, 1998), intracellular cAMP levels in

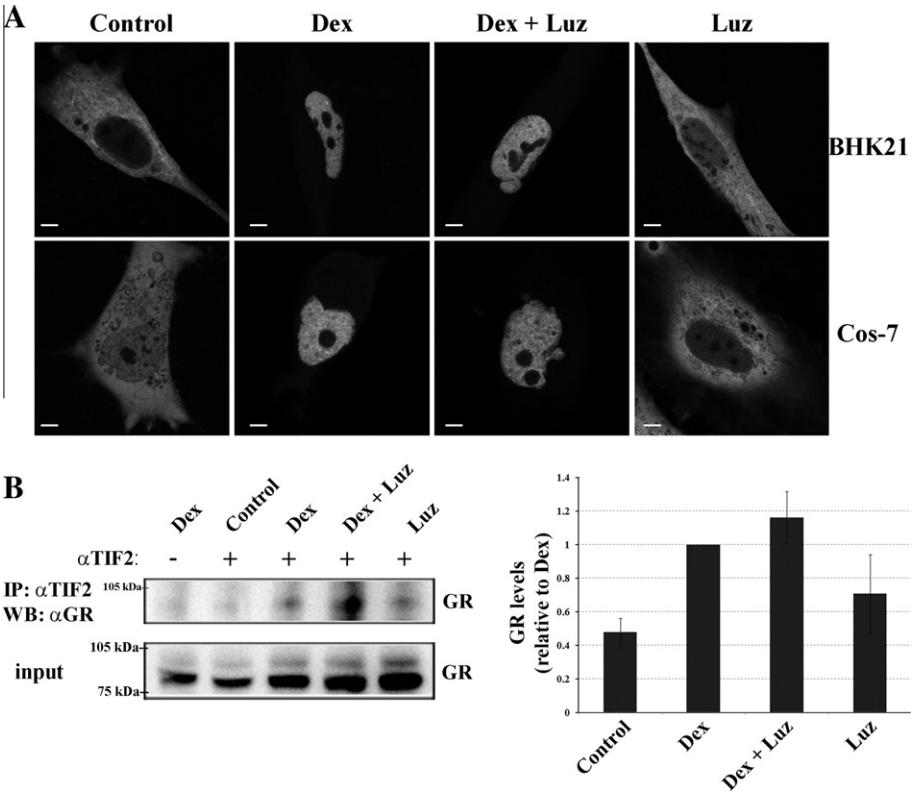

Fig. 8. Luzindole affects neither GR nuclear translocation nor TIF2–GR association. A. BHK21 and Cos-7 cells transfected with pEGFPGR were incubated with the indicated hormones. The figure shows one representative cell for each treatment. Scale bar = 20 μm. B. BHK21 cells co-transfected with pRSV-hGR and pTIF2 vectors were incubated with the indicated hormones for 90 min. Immunoadsorptions were performed as described in Fig. 4. The figure shows gels corresponding to one representative experiment ($n = 3$) and Mean ± SE values of immunoprecipitated (IP) GR levels (respect to Dex treatment).

BHK21 cells were determined by radioimmunoassays. DEX decreased cAMP levels (Fig. 7, Dex vs. Control) as it was previously reported on other cellular types (Dong et al., 2004; Hoijman et al., 2004; Iwasaki et al., 1997). Melatonin had neither effect *per se* nor antagonized GC action (Fig. 7). However, in the presence of luzindole, DEX effect was reverted (Fig. 7, LUZ vs. Dex). On one hand, these results indicate that cAMP-dependent pathway would not be involved on GR-melatonin antagonism. On the other hand, since luzindole inhibits both GR-dependent transcriptional activity and cAMP downregulation, we postulate luzindole as a putative antiglucocorticoid.

In order to understand the mechanism by which luzindole antagonizes GC effects, we next analyzed GFPGR sub-cellular localization in BHK21 and Cos-7 cells. As shown in Fig. 8A, luzindole does not impair DEX-induced GR nuclear translocation; nor promotes its nuclear import in the absence of DEX. Thus, similarly to melatonin, luzindole would block GR action at some point downstream nuclear import. Therefore, GR–TIF2 interaction was then analyzed by performing Co-IP assays from extracts treated with luzindole. Fig. 8B shows that luzindole – unlike melatonin – does not prevent GR–TIF2 association.

4. Discussion

As stated before, melatonin antagonism on GR action has been reported in several works. However, most studies did not clarify about the mechanism by which melatonin impairs GR activation: i.e. does melatonin affect GR–ligand binding? Does it impair GR-Hsp90 dissociation? May melatonin block GR nuclear translocation? Does it affect GR–DNA interaction or does it impede GR homodimerization or GR co-activators recruitment? These questions still were not fully assessed. In a previous work we have demonstrated in mouse thymocytes that melatonin inhibits GR nuclear translocation, most likely by blocking GR–Hsp90 dissociation (Presman et al., 2006). Similar results were also reported in the neuron HT22 cell line model (Quiros et al., 2008). Here we found that melatonin has the ability to inhibit GR-dependent MMTV-driven gene expression on BHK21 cells, but surprisingly this antagonistic effect does not involve modulation on GR sub-cellular localization nor intranuclear distribution. In fact, melatonin even seems to affect neither GR homodimerization *in vivo* nor GR–DNA interaction *in vitro*. Interestingly, Co-IP results suggest that dexamethasone-dependent GR–TIF2 interaction is compromised in the presence of the methoxyindole. Since it has been demonstrated that GR-dependent MMTV induction drastically decreases when TIF2 is down-regulated (Li et al., 2003); results presented here suggest that although GR may still be able to bind GRE elements in BHK21 cells, it would be unable to induce the expression of target genes due to its inability to recruit TIF2. In this sense, it is possible that melatonin would also modulate the recruitment of another cofactor or even influence the role of any of the GR-associated transcription factors such as NFκB, AP-1 or STATs. Together, these results suggest that melatonin antagonizes glucocorticoids by different mechanisms according to cell type. The modulation of subcellular localization (thymocytes or neuronal cells) or cofactor recruitment impairment (BHK21 cells) could account for at least two possibilities.

How can melatonin antagonize glucocorticoids at different points of the GR activation pathway? Assuming that melatonin indirectly acts on the glucocorticoid receptor, different signaling pathways would be involved on melatonin effect depending on each cell type. In this sense, both nuclear and membrane receptors could mediate melatonin signaling (Reiter et al., 2010). As stated before, MT_1 and MT_2 receptors act mainly through a cAMP-dependent signaling pathway (Vanecek, 1998). The fact that melatonin does not antagonize glucocorticoids effect on cAMP levels in both thymocytes (Hoijman et al., 2004) and BHK21 cells (Fig. 7), indicates that MTs involvement on melatonin-glucocorticoid antagonism would be a highly unlikely event. On the other hand, cell specific expression of proteins involved on GR signaling pathway could explain melatonin dependent cell-type differential response. In this sense, it has been proposed that GR–Hsp90 interaction with a ligand-bound GR mimics the receptor's interaction with transcriptional co-activators (De Bosscher and Haegeman, 2009). In this context, if melatonin directly or indirectly modulates GR conformation on thymocytes (i.e. leading to the regulation of the GR–Hsp90 heterocomplex), it is also possible that a similar mechanism could explain the lack of GR–TIF2 interaction on BHK21 cells. Nevertheless, further studies focused on the docking of different proteins on a specific GR domain should be performed.

Luzindole is a well-known antagonist of MT receptors. In fact, this molecule presents high affinity for MT_1 and MT_2 (Boutin et al., 2005). Despite the extensive use of luzindole, many pharmacological characteristics of this substance still remain unknown (Mathes et al., 2008). For example, it has been shown dose-dependent variations in its response on the rat caudal artery (Masana et al., 2002); partial agonistic action on HL-60 leukemic cells (Cabrera et al., 2003) and an antioxidant activity *in vitro* (Mathes et al., 2008). With the original objective of testing MT receptor's involvement on the melatonin-GC antagonism, we tested luzindole effect on melatonin action. Surprisingly, we found that this compound also present an antagonistic effect on GR action. Here, we demonstrated that luzindole is able to inhibit the glucocorticoid-dependent expression of an MMTV-driven reporter gene in a dose-dependent manner and also to reduce the activity of the glucocorticoid-sensitive BclX.P4 reporter gene. Moreover, opposite to melatonin behavior, luzindole was able to prevent DEX-dependent downregulation of cAMP levels in BHK21 cells and to inhibit MMTV activity on Cos-7 cells. Involvement of MT receptors on luzindole effect is highly unlikely because Cos-7 cells do not express any melatonin receptors (Roca et al., 1996).

Regarding luzindole mechanism of action, we found no effect on DEX-dependent GR nuclear translocation. Since this process occurs upon DEX-binding even in the presence of luzindole, this fact strongly suggests that this methoxyindol does not affect DEX ability to bind GR. Strikingly; luzindole has no detectable effect on DEX-dependent GR–TIF2 interaction on BHK21 cells. This result suggests that this compound – also differing from melatonin – would affect an event downstream to GR cofactor recruitment. Nonetheless, the fact that no additive effects on GR antagonism was observed when relatively low concentrations of both methoxyindoles were used, suggests that a more complex mechanism would be implicated on the effect of either compound.

In conclusion, results presented here indicate that melatonin would modulate GR activity by different mechanisms and suggest luzindole as a potentially new antiglucocorticoid.

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