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Melatonin, an Endogenous-specific Inhibitor of Estrogen Receptor α via Calmodulin*

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Melatonin is an indole hormone produced mainly by the pineal gland. We have previously demonstrated that melatonin interferes with estrogen (E_2) signaling in MCF7 cells by impairing estrogen receptor (ER) pathways. Here we present the characterization of its mechanism of action showing that melatonin is a specific inhibitor of E_2-induced ERα-mediated transcription in both estrogen response element- and AP1-containing promoters, whereas ERβ-mediated transactivation is not inhibited or even activated at certain promoters. We show that the sensitivity of MCF-7 cells to melatonin depends on the ERα/ERβ ratio, and ectopic expression of ERβ results in MCF-7 cells becoming insensitive to this hormone. Melatonin acts as a calmodulin antagonist inducing conformational changes in the ERα-calmodulin (CaM) complex, thus impairing the binding of E_2·ERα·CaM complex to DNA and, therefore, preventing ERα-dependent transcription. Moreover the mutant ERα (K302G,K303G), unable to bind calmodulin, becomes insensitive to melatonin. The effect of melatonin is specific since other related indoles neither interact with CaM nor inhibit ERα-mediated transactivation. Interestingly, melatonin does not affect the binding of coactivators to ERα, indicating that melatonin action is different from that of current therapeutic anti-estrogens used in breast cancer therapy. Thus, they target ERα at different levels, representing two independent ways to control ERα activity. It is, therefore, conceivably a synergistic pharmacological effect of melatonin and current anti-estrogen drugs.

Melatonin is an indole hormone that is the major secretory product of the pineal gland. The most clearly defined actions of melatonin have been demonstrated on the reproductive system of seasonally breeding animals and on circadian rhythms and sleep. A rapidly emerging avenue of investigation is the oncostatic and anti-proliferative effects of melatonin on endocrine-responsive neoplasms, especially in those concerning the mammary gland (1). The most common conclusion in animal models of tumorigenesis is that either experimental manipulations that activate the pineal gland or the administration of melatonin reduces the incidence and development of chemically induced mammary tumors, whereas pinealectomy usually stimulates breast cancer growth (2–4). Epidemiological studies have shown a low incidence of breast tumors in blind women as well as an inverse relationship between breast cancer incidence and the degree of visual impairment. Because light inhibits melatonin secretion, the increase in melatonin-circulating levels might be interpreted as proof of the protective role of this hormone on mammary carcinogenesis (5). A moderate increase in breast cancer risk among women who worked extended periods of rotating night shifts (light exposure during night suppresses melatonin production) has also been described (6).

Different mechanisms have been proposed to explain how melatonin could reduce the development of mammary tumors; they are indirect neuroendocrine mechanisms such as melatonin regulation of some pituitary and gonadal hormones that control tumor growth (1, 4–7) and the direct effects of melatonin as an endogenous hydroxyl radical scavenger (8) or as a modulator of the immune response to the presence of a malignant neoplasm (9, 10). On the other hand, direct anti-estrogenic melatonin actions at the cellular level have been proposed (11, 12). Studies using MCF-7 human breast cancer cells (ER+)[1] (an estrogen-dependent model system, as is the case for more than 60% of primary breast tumors) demonstrate that physiological concentrations of melatonin (1 nM to 1 pM) exert a direct anti-proliferative effect on estrogen-induced proliferation of these cells (11, 12) and reduce their invasiveness, causing a decrease in cell attachment and cell motility, probably by interacting with estrogen-mediated mechanisms (13). However, the molecular basis of melatonin action remains largely unknown.

In a previous report (14), we presented evidence that melatonin interferes with estrogen-signaling pathways. We demonstrated that melatonin acts as anti-estrogen by preventing the estrogen-dependent transcriptional activation in MCF-7 cells through destabilization of the E_2·ER complex from binding to DNA, and we proposed calmodulin (CaM) as a potential candidate for mediating the anti-estrogenic effects of melatonin. Several lines of evidence support this hypothesis; the interaction of this calcium-regulated protein with ER has been known for several years, and a number of CaM antagonists exhibit anti-estrogenic activity and decrease the affinity of ERα for its ligand as well as the stability of E_2·ER binding to DNA (15). In addition, it has been shown that melatonin binds to calmodulin in a Ca^{2+}-dependent fashion, resulting in the inhibition of calmodulin (16, 17).

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[1] The abbreviations used are: ER, estrogen receptor; ERE, estrogen response element; E_2, estrogen; CaM, calmodulin; GST, glutathione *S*-transferase; MOPS, 4-morpholinepropanesulfonic acid; EGF, epidermal growth factor; TFP, trifluoperazine; OHT, 4-hydroxytamoxifen.

In the search for differences between ERα and the most recently described ERβ, we analyzed the interaction of both receptors with calmodulin, and we demonstrated that ERα but not ERβ directly interacts with calmodulin. Consequently, CaM antagonists are selective modulators of ERα-mediated transcription (18). In the present study, we have investigated whether calmodulin could be a link between melatonin and the estrogen-signaling pathway. Our results indicate that melatonin acts as specific inhibitor of ERα at physiological doses, and therefore, clinical studies on the possible therapeutic value of melatonin on breast cancer should be considered.

EXPERIMENTAL PROCEDURES

Materials—Melatonin, 17β-estradiol, 4-hydroxytamoxifen, and other chemicals were purchased from Sigma. ICI 182,780 was provided by Dr. A. E. Wakeling (Zeneca Pharmaceuticals, Macckesfield, Cheshire, UK). [^{35}S]Methionine (Pro-mix; 14.3 mCi/ml; >1000 Ci/mmol) was from Amersham Biosciences.

Plasmids—The expression vector pcDNA-ERα and the recombinant plasmid GST-ERα-(280–595) have been previously described (18, 19). pERE-TK-Luc, pS2-Luc, and pCMX-mERβ were kindly provided by Dr. V. Giguère from the R. W. Johnson Pharmaceutical Research Institute, Don Mills, Ontario, Canada. pCXN2-hERβ-(1–530), GST-hERα-(117–595) (20), and pRL-TK (Promega Corp., Madison, WI) were also used in this work. The plasmid 3x-ERE-TATA-Luc was kindly provided by Dr. S. Safe from the Department of Veterinary Physiology and Pharmacology, Texas A&M University, College Station, TX. Δcoll-73 was kindly provided by Dr. A. Aranda from Instituto de Investigaciones Biomédicas "Alberto Sols" Consejo Superior de Investigaciones Científicas, Madrid, Spain.

Cell Culture and Transient Transfection Assays—HeLa cells were propagated as previously described (18). Before transfection, HeLa cells were seeded in 12-well plates and incubated for 12–18 h at 37 °C. Then cells were transferred to phenol red-free Dulbecco's modified Eagle's medium containing 0.5% charcoal, dextran-treated fetal calf serum and maintained for 3 days. At 60–80% confluency, cells were transfected with 0.5 μg of estrogen response element (ERE)-driven or AP1-driven reporter plasmids, 0.1 μg of ER expression vector, and 50 ng of an internal control *Renilla* luciferase plasmid, pRL-TK (Promega), using FuGENE 6 transfection reagent from Roche Applied Science following the manufacturer's protocols. After 18–24 h, medium was renewed, and cells were stimulated for 24 h with different chemicals as indicated.

Luciferase was assayed with the dual luciferase system (Promega). Luciferase activities were normalized to *Renilla* luciferase activity to correct for differences in transfection efficiency. The results represent the means ± S.D. of three independent experiments performed at least in duplicate. Transactivation experiments were performed with both mouse and human ERβ, and identical trends in ligand behavior were observed in both ERβs in HeLa cells.

MCF-7 cells were propagated in RPMI 1640 medium containing 25 mM HEPES, NaOH, pH 7.3, and synchronized cells were transfected as above. When indicated, ERβ expression vector or the empty vector was included in the transfection.

Electrophoretic Mobility Shift Assay—Binding of the E_2·ER to ERE was performed as previously described (14). Five to ten microliters of nuclear extracts of transient transfections were mixed with buffer B (20 mM HEPES-KOH, pH 7.9, 10 mM $MgCl_2$, 1 mM EDTA, 10% (v/v) glycerol, 100 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride, 0.2 mM dithiothreitol, 0.5% Nonidet P-40, and protease inhibitors) and incubated with 1 μg of poly(dI·dC) in a total volume of 40 μl. Mixtures were preincubated at 0 °C for 15 min followed by incubation with the indicated hormones at 0 °C for 10 min. ^{32}P-Labeled probe (10 fmol containing 3–5 × 10^4 dpm) corresponding to the ERE from *Xenopus* vitellogenin A_2 gene was added to the reaction and allowed to proceed for 1 h at 0 °C followed by 30 min at room temperature. The samples were loaded onto a pre-electrophoresed (10 mA) 5% polyacrylamide gel (acrylamide to bisacrylamide ratio of 40:1) in TBE (45 mM Tris borate, 1 mM EDTA) at 11 mV/cm. Gels were vacuum-dried and exposed at −80 °C to obtain the autoradiography. For specificity assays, a 100-fold excess of unlabeled oligonucleotide was used as competitor before adding the probe to the binding reaction.

Proteolysis Assays—The pcDNA-ERα plasmid (1 μg) containing full-length cDNA of the wild-type human ERα was used to produce ^{35}S-radiolabeled ERα using 40 μl of a coupled transcription-translation system according to the manufacturer's instructions (Promega). The protease digestion was performed essentially as described by McDonnell *et al.* (21). An aliquot (4.5 μl) of reticulocyte lysate was incubated for 20 min in the absence or presence of 1 μM 17β-estradiol, 1 nM melatonin, and 1 μM W7 as indicated. Equal aliquots of the untreated or the hormone-treated receptor were subsequently incubated with a trypsin solution (Roche Applied Science), giving final enzyme concentration of 25 μg/ml. After 10 min of incubation at room temperature, the digestion reaction was terminated by the addition of gel-denaturing buffer and boiling for 5 min. The products of the digestion procedure were separated on a 12% polyacrylamide-SDS gel. After electrophoresis the gel was treated with a 0.5 M sodium salicylate solution for 15 min. The gel was dried under vacuum, and the radiolabeled products were visualized by autoradiography. When indicated, 1 μg of goat polyclonal anti-CaM antibodies (SC-1988, Santa Cruz Biotechnology, Inc.) was added before the treatment with hormones.

In Vitro Protein-Protein Interaction Assays—GST fusion proteins were expressed and purified essentially as described by Frangioni and Neel (22). GST pull-down experiments were performed as previously described by Cavailles *et al.* (23). ^{35}S-Labeled SRC-1a coactivator was synthesized by *in vitro* transcription-translation (Promega) using pCR-SRC-1a as template. The GST fusion proteins loaded on glutathione-Sepharose beads (25 μl) were preincubated with 1 μM concentrations of ligands (17β-estradiol, 4-hydroxytamoxifen, or ICI 182,780) or 1 nM melatonin for 30 min at 4 °C followed by incubation with ^{35}S-labeled proteins for 1.5 h at 4 °C in a total volume of 150 μl of IPAB buffer (20 mM HEPES-KOH, pH 7.9, 5 mM $MgCl_2$, 150 mM KCl, 0.02 mg/ml bovine serum albumin, 0.1% (v/v) Triton X-100, 0.1% Nonidet P-40, and protease inhibitors). Beads were washed 4–5 times with IPAB without bovine serum albumin, collected by centrifugation, and resuspended in 20 μl of loading buffer for SDS-PAGE analysis. The gel was vacuum-dried, and the radiolabeled products were visualized by autoradiography.

In Vitro Interaction with Dansyl-CaM—Fluorescence experiments were performed in a PerkinElmer Life Sciences fluorimeter using a 100-μl cuvette. 2.5 nmol of dansyl-CaM (Sigma) were dissolved in 100 μl of 10 mM MOPS, pH 7.2, 1 mM $MgCl_2$, 100 mM KCl, and 1 mM $CaCl_2$. Emission fluorescent spectra were obtained (λ_{Ex} 333 nm) before and after the addition of 1 nM melatonin or the indole derivatives. Equivalent amounts of buffer were added in the controls.

RESULTS

Melatonin Is a Specific Inhibitor of ERα-mediated Transcription—We have previously demonstrated that melatonin is able to inhibit estrogen-dependent transcription and proliferation in MCF-7 cells (14). Because MCF-7 is a carcinoma-derived cell line (ER+) that endogenously expresses both ERα and ERβ, we further investigated the inhibitory effect of melatonin on E_2-dependent transactivation mediated by each receptor isoform independently. For this purpose, we transiently transfected HeLa cells with either ERα or ERβ expression vectors along with the ERE-driven reporter plasmids pEREtkLuc (Fig. 1*A*), pS2Luc (Fig. 1*B*), or 3xERELuc (Fig. 1*C*). In all cases 10 nM E_2 stimulated transcription for both ERα- and ERβ-transfected cells. As expected, the highest E_2 stimulation was obtained using a strong promoter containing three ERE sites (Fig. 1*C*). Physiological concentrations of melatonin (1 nM) inhibited ERα-mediated transactivation by 45–60% depending on the promoter tested. In contrast, ERβ-mediated transcription was not affected (Fig. 1, *B* and *C*) or even potentiated (Fig. 1*A*) by this concentration of melatonin. In titration experiments we observed that melatonin inhibited ERα-mediated transcription in a dose-dependent manner, whereas ERβ activity was unaffected by the different concentrations of melatonin assayed (Fig. 1*D*). These results indicate that melatonin is a selective modulator of ERα, as we have recently described for CaM antagonists (18).

An ERα Mutant Unable to Bind CaM Is Insensitive to Melatonin—In a previous report we demonstrated that residues Lys-302 and Lys-303 of hERα are essential for CaM binding. Although the wild-type ERα normally binds to CaM, substitution of lysines 302 and 303 by glycine abolished the interaction of ERα (K302G,K303G) with CaM (18). Transcriptional activation studies further demonstrated that these two critical resi-

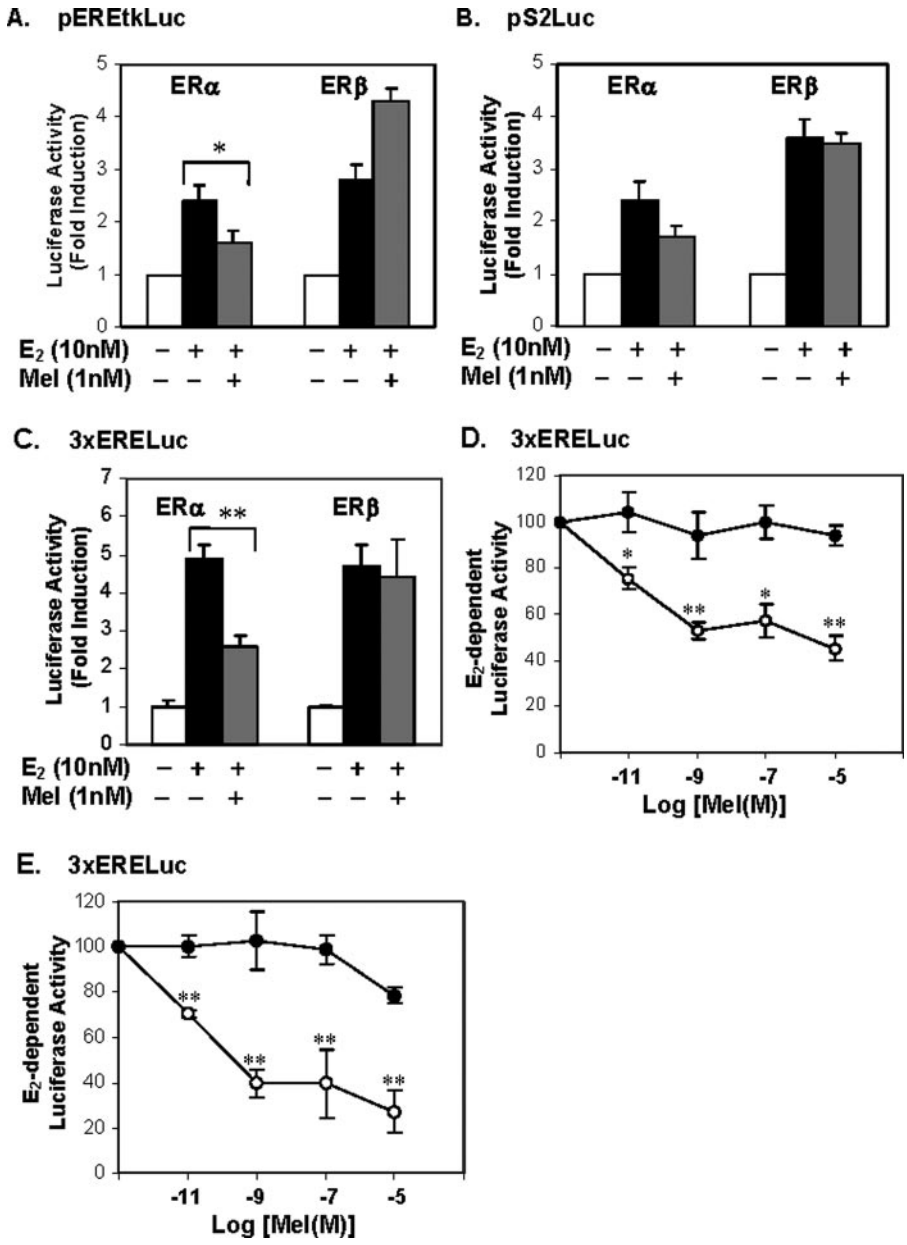

FIG. 1. **Differential effect of melatonin on the transactivation properties of ERα and ERβ.** HeLa cells were transfected with 0.1 μg of ERα or ERβ expression vectors, 50 ng of the internal control plasmid pRL-TK, and 0.5 μg of the following ERE-driven reporter plasmids pEREtkLuc (*A*), pS2Luc (*B*) or 3xERELuc (*C*). After 18–24 h, medium was renewed, and cells were stimulated for 24 h with 10 nM E_2 and 1 nM melatonin (*Mel*) as indicated. Luciferase activities were normalized to the *Renilla* luciferase activities. The data are reported as fold induction relative to untreated cells, which were arbitrarily assigned as 1. The *bars* represent the mean ± S.D. of three independent experiments performed in duplicate. The *asterisk* indicates a statistical significant difference (Student's *t* test), $p < 0.05$, between the groups. *D*, dose response to melatonin of E_2-induced ERα (○) or ERβ (●) transactivation. HeLa cells were processed as above and treated with 10 nM E_2 and the indicated concentrations of melatonin. E_2-dependent luciferase activity is expressed as the percentage of E_2 stimulation. The *bars* represent the means ± S.D. of three independent experiments run in duplicate. These results were significantly different from E_2-stimulated transcription: *, $p < 0.05$; **, $p < 0.01$. *E*, dose response to Mel of E_2-induced ERα (○) or ERα (K302G,K303G) (●) transactivation. HeLa cells were transfected with 0.5 μg of 3xERELuc reporter plasmid, 50 ng of the internal control plasmid pRL-TK, and 0.1 μg of either ERα or ERα (K302G,K303G) expression vectors, and cells were treated with 10 nM E_2 and the indicated concentrations of melatonin. These results are significantly different than E_2-stimulated transcription: *, $p < 0.05$; **, $p < 0.01$.

dues for ERα binding to CaM are not essential for ERα transcriptional activation. Thus, when HeLa cells were transiently transfected with wild-type ERα and compared with those transfected with ERα (K302G,K303G), both showed similar levels of basal and E_2-induced transcriptional activation (18). However, ERα transactivation was 80% inhibited by 10^{-6} M W7, whereas transcription mediated by ERα (K302G,K303G) was completely insensitive to this calmodulin antagonist. If melatonin acts as a calmodulin antagonist on ERα-mediated transcription, we could predict no inhibitory effect of the pineal hormone on ERα (K302G,K303G)-mediated transactivation. Indeed, when HeLa cells were transiently transfected with ERα (K302G,K303G) along with the ERE-driven reporter plasmid 3xERELuc (Fig. 1*E*) we observed that ERα (K302G, K303G) transcription was unaffected by the different concentrations of melatonin assayed.

The Sensitivity of MCF7 Cells to Melatonin Depends on the ERα/ERβ Ratio—In MCF7 cells increasing concentrations of melatonin resulted in the progressive inhibition of the E_2-dependent transcription, reaching nearly 100% of inhibition at pharmacological concentrations of melatonin (Fig. 2*A*). The IC_{50} was obtained at 1.26×10^{-11} M as determined with GraphPad Prism.

We next analyzed whether the sensitivity of MCF7 cells to inhibition by melatonin was associated with the high ERα/ERβ ratio present in these cells, as we have previously reported for CaM antagonists (18). To test this hypothesis, MCF7 cells were transfected with the 3xERELuc reporter plasmid in the absence or presence of an ERβ expression vector. We then determined whether ERβ overexpression affects the sensitivity of these cells to melatonin and compared its effect to those of the CaM antagonists W7 and calmidazolium. As expected, both melatonin and CaM antagonists inhibited E_2-dependent transcriptional activation in MCF7 cells (Fig. 2*B*). Interestingly, the inhibitory effects of both melatonin and CaM antagonists were abolished by ERβ overexpression (Fig. 2*B*). These results imply that the sensitivity to melatonin of E_2-induced transcription in MCF7 cells depends on the presence of ERα. Inhibition by melatonin correlates with a high ERα/ERβ ratio, whereas an increased expression of ERβ impairs the effect of the hormone.

Melatonin Inhibits E_2·ERα-mediated Transcription in AP1-containing Promoters—We have previously demonstrated that

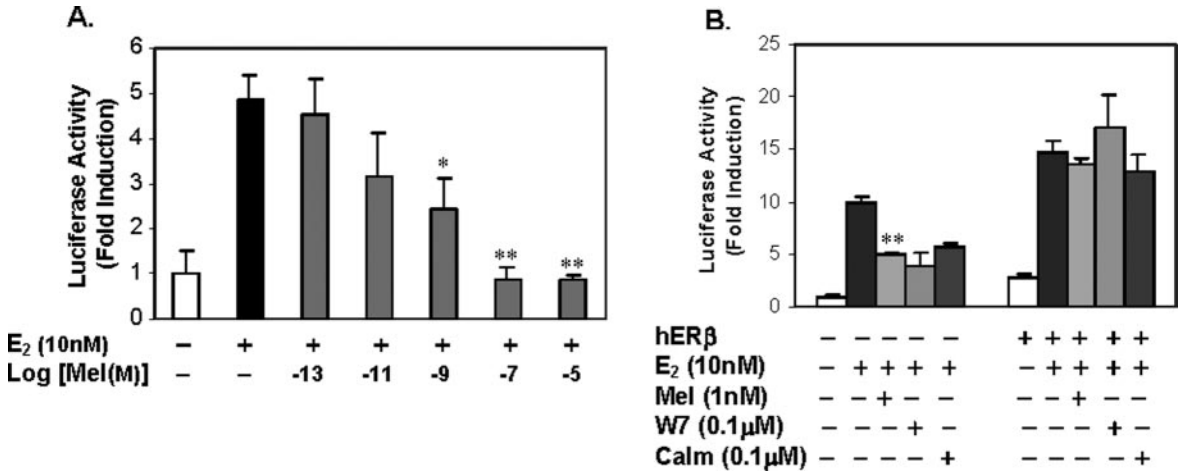

FIG. 2. **The sensitivity of MCF7 cells to melatonin and CaM antagonists is abolished by ERβ overexpression.** *A*, dose response to melatonin of E_2-dependent transactivation in MCF-7 cells. Transfections were performed by using 0.5 μg of 3xERELuc reporter plasmid, and after 18–24 h, cells were stimulated for 24 h with either 10 nM E_2 alone or 10 nM E_2 plus the indicated concentrations of melatonin. An IC_{50} value of 1.26×10^{-11} M was generated by non-linear regression analysis using GraphPad Prism, version 3.02 for windows (GraphPad Software, Inc., San Diego, CA). *B*, MCF-7 cells were transfected with 0.5 μg of the reporter plasmid 3xERELuc, 50 ng of pRL-TK plasmid, and when indicated, with 0.4 μg of ERβ expression vector (*right set* of *data*). The total amount of DNA was held constant to 0.95 μg per well by the addition of empty expression vector. After transfection, cells were treated for 24 h with 10 nM E_2, 1 nM melatonin (*Mel*), 0.1 μM W7, or 0.1 μM calmidazolium (*Calm*) as indicated. Luciferase activities were normalized to the *Renilla* luciferase activities. The data are reported as -fold induction relative to untreated cells, which were arbitrarily assigned as 1. The *bars* represent the means ± S.D. of three independent experiments performed in duplicate. These results are significantly different than E_2-stimulated transcription: *, $p < 0.05$; **, $p < 0.01$.

CaM is a regulator of ERα-mediated transcription in both ERE- and AP1-containing promoters since transcription mediated by both complexes is sensitive to CaM antagonists (18). Therefore, we decided to test the ability of melatonin to inhibit transcription on ERα/AP1 pathways. For that purpose, HeLa cells were transfected with either ERα or ERβ along with the reporter plasmid Δcoll-73-Luc (containing an AP1 binding site). Even though E_2·ERα-mediated AP1 activation in HeLa cells and other cell lines have been described (24, 25), we and other authors found it necessary to prime the cells with EGF to observe this effect. EGF stabilizes the levels of c-Jun and c-Fos family proteins, allowing a synergistic effect between these factors and ERα on AP1 transcription (26, 27). We found that AP1 activity was increased by EGF in cells expressing either ERα or ERβ (Fig. 3). E_2 significantly potentiated AP1 activity in ERα-transfected cells but diminished AP1 activity in ERβ-transfected cells. These results agree with previous reports (26, 27) indicating that EGF synergizes with E_2. Very importantly, the synergistic effect of EGF and E_2 in cells expressing ERα was sensitive to melatonin, whereas no effect was observed in cells expressing ERβ. Both the activation by E_2 and the inhibition by melatonin were statistically significant. We can infer from these experiments that melatonin, as other CaM antagonists, regulates ERα-mediated transcription not only in ERE-dependent pathways but also in AP1 pathways.

Melatonin but Not Other Indole Derivatives Interact with CaM—We have previously demonstrated that melatonin blocks the binding of the E_2·ER complex to ERE *in vitro* and that this effect is dose-dependent, saturable, and specific, since different methoxy- and hydroxyindoles have no effect on binding to DNA (14). Therefore, we expected that other indole derivatives would have no effect on E_2·ERα-mediated transcription. To analyze this possibility we carried out transient transfections in MCF-7 cells using 3xERE-Luc as reporter plasmid. As shown in Fig. 4*B*, melatonin effectively inhibited (60%) E_2-induced transactivation, whereas treatment with other indole metabolites resulted in no significant decrease on the E_2-mediated transcription, indicating that the inhibitory effect of melatonin on estrogen response is specific.

To further investigate the basis for the specific inhibition exhibited by melatonin, we examined the ability of the indole derivatives to bind to dansyl-CaM. Changes on emission fluorescence intensity of dansyl-CaM reflect conformational/structural changes, suggesting interaction with CaM. As observed in Fig. 4*C*, melatonin specifically decreased the fluorescence of dansyl-CaM, whereas the other indoles tested did not modify the fluorescence of dansyl-CaM, indicating that only melatonin is able to interact with this protein. We hypothesize that melatonin acts as a CaM antagonist, interfering with the ERα-CaM complex and that this is the underlying basis by which melatonin specifically inhibits ERα-mediated transcription.

Melatonin Induces Conformational Changes on ERα Structure via CaM—Conformational changes on ERα structure can be shown by using a protease digestion assay as previously described by McDonnell *et al.* (21). Also, W7 induces CaM to form a globular structure (29). We have determined the effects of melatonin and W7 on ERα structure on the basis of the differential susceptibility of the receptor to proteolysis by trypsin. ^{35}S-Labeled ERα was synthesized *in vitro* and preincubated with vehicle, E_2, melatonin, W7, or combinations of these compounds. The resulting complexes were then subjected to limited digestion with trypsin, and the products were resolved by SDS-PAGE (Fig. 5). ERα was highly sensitive to trypsin degradation in the absence of ligand (Fig. 5, *lane 2*), whereas in the presence of E_2, a trypsin-resistant 32-kDa fragment was observed (Fig. 5, *lane 4*), in agreement with results previously published (21). Incubation of the labeled receptor in the presence of E_2 plus either melatonin (Fig. 5, *lane 6*) or W7 (Fig. 5, *lane 8*) yielded a distinct digestion patron as compared with E_2 alone. Under these conditions, ERα becomes highly sensitive to protease digestion. Therefore, treatment with melatonin or CaM antagonists abolished the protective effect of E_2 on limited trypsin digestion. Interestingly, the effects of melatonin (Fig. 5, *lane 5*) and W7 (Fig. 5, *lane 7*) were reverted in the presence of anti-CaM antibodies. Taken together, our data indicate that melatonin, similar to W7 through the interaction with CaM, induce conformational changes on ERα that also affect the stability of ERα against proteolysis.

ERα Stability Is Not Altered by Melatonin—It has been reported that the inhibition of the interaction between CaM and

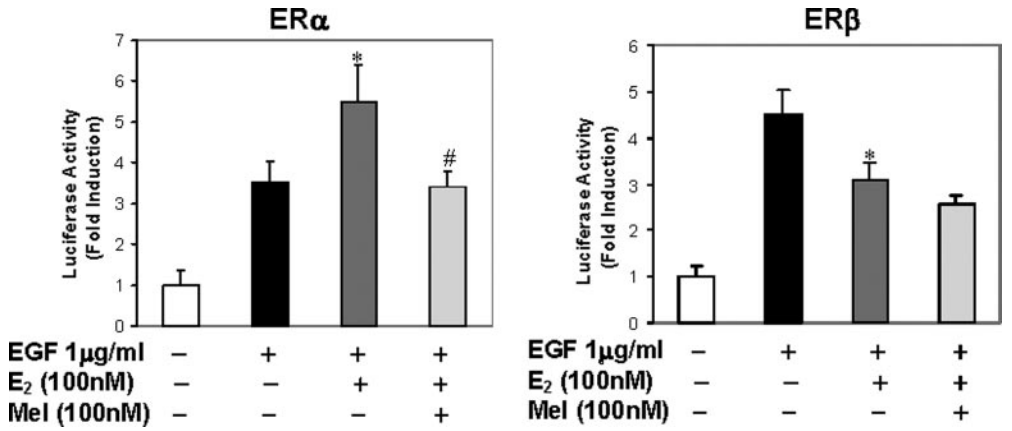

FIG. 3. **Effect of melatonin on E_2-dependent transactivation at an AP1 element.** HeLa cells were transfected with either 0.1 μg of the ERα or ERβ expression vectors, 50 ng of internal control plasmid pRL-TK, and 0.5 μg of the AP1-containing reporter plasmid (Δcoll-73-Luc). Cultures were stimulated for 48 h with 1 μg/ml EGF, 100 nM E_2, and 100 nM melatonin (*Mel*) as indicated. The data are reported as –fold induction relative to untreated cells, which were arbitrarily assigned as 1. The *bars* represent the means ± S.D. of three independent experiments run in duplicate. *, significantly different relative to EGF treatment ($p < 0.05$). #, significant relative to EGF plus E_2 treatment ($p < 0.05$).

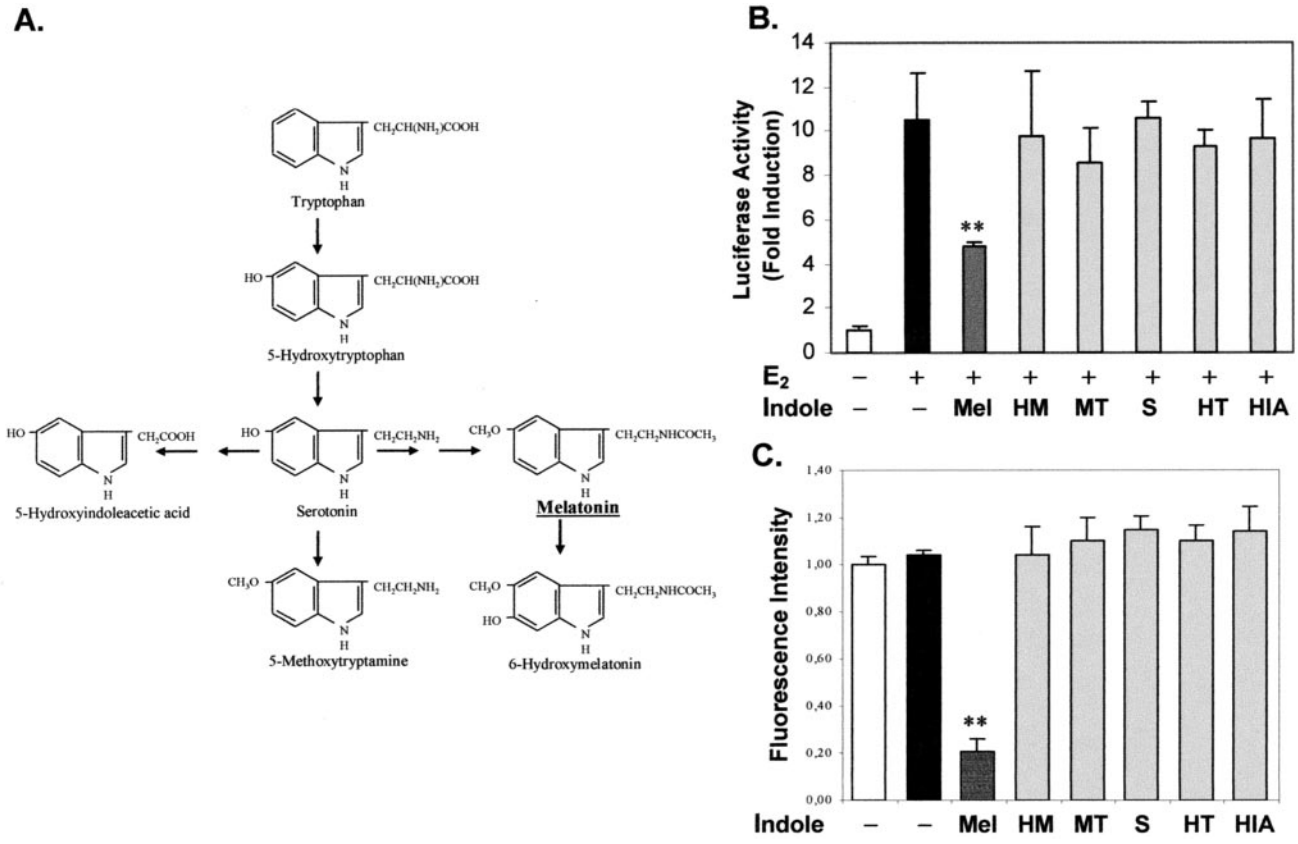

FIG. 4. **Effect of different hydroxy- and methoxyindoles on ERα-mediated transcription and interaction with dansyl-CaM.** *A*, schematic diagram of the indole derivatives implicated in the metabolism of melatonin. *B*, HeLa cells were transfected with 0.1 μg of the ERα expression vector, 50 ng of internal control plasmid pRL-TK, and 0.5 μg of 3xERELuc as ERE-driven reporter plasmid. After 18–24 h, medium was renewed, and cells were stimulated for 24 h with 10 nM E_2 or 10 nM E_2 plus either 1 nM melatonin (*Mel*), 6-hydroxymelatonin (*HM*), 5-methoxytryptamine (*MT*), serotonin (*S*), 5-hydroxytryptophan (*HT*), or 5-hydroxyindole acetate (*HIA*) as indicated. Luciferase activities were normalized to the *Renilla* luciferase activities. The data are reported as fold induction relative to untreated cells, which were arbitrarily assigned as 1. The *bars* represent the means ± S.D. of three independent experiments run in duplicate. Significant difference from E_2-stimulated transcription: **, $p < 0.01$. *C*, ability of the different indoles to bind dansyl-CaM. Emission fluorescence spectra of 25 μM dansyl-CaM in 10 mM MOPS (pH 7.2), 1 mM $MgCl_2$, 100 mM KCl, and 1 mM $CaCl_2$ were determined, and this intensity was arbitrarily assigned the value 1. 1 nM melatonin, 6-hydroxymelatonin, 5-methoxytryptamine, serotonin, 5-hydroxytryptophan, or 5-hydroxyindole acetate was added, and fluorescence was determined after the addition of each compound. The *bars* represent the means ± S.D. of two independent experiments performed in duplicate. **, significant relative to untreated cells; $p < 0.01$.

ER reduces the total cellular content of estrogen receptor (28). Thus, treatment of MCF7 cells with calmodulin antagonists such as trifluoperazine (TFP) or CGS9343B reduced the number of estrogen receptors in the cells (30). We have addressed whether the inhibition of ERα activity by melatonin in MCF7 cells could be due to the modulation of the stability and the state level of estrogen receptors. For this purpose, MCF7 cells were treated for 24 h with either vehicle, E_2, melatonin, TFP, or combinations, and ER protein levels were determined by Western blot. As shown in Fig. 6, E_2 (*fourth lane*) and TFP (*fifth*

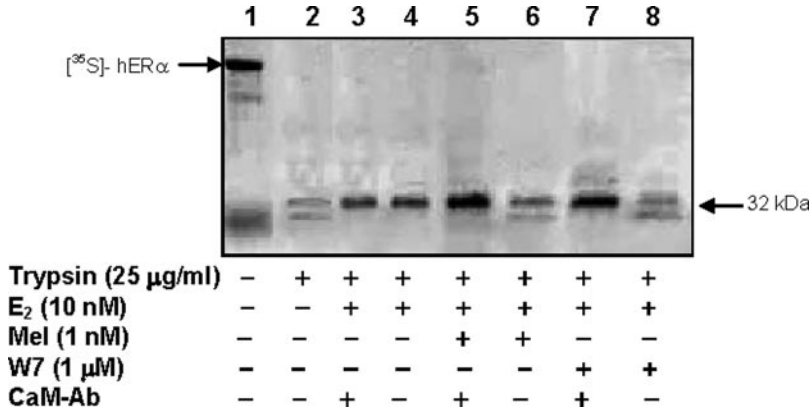

FIG. 5. **Effect of melatonin and W7 on the proteolysis of ERα.** *In vitro* translated ^{35}S-labeled ERα was subjected to digestion with 25 μg/ml of trypsin in the absence or presence of 10 nM E_2, 1 nM melatonin (*Mel*), 1 μM W7, and 1 μg of polyclonal anti-CaM antibodies (*Ab*) as indicated. The products of the digestion reactions were resolved by SDS-PAGE and visualized by autoradiography. *h-*, human.

lane) significantly reduced ERα content in MCF7 cells, in agreement with previous data (30). Strikingly, treatment with 100 nM melatonin does not alter the amount of ERα in the cells (Fig. 6, *second lane*), indicating that inhibition of ERα-mediated transcription by melatonin is not due to a reduction in ER protein levels. Importantly, neither TFP nor melatonin affected ERβ levels, suggesting that the interaction with CaM is important to trigger degradation since ERα but not ERβ interacts with calmodulin.

Effect of Melatonin on Coactivator Binding Properties of ERα—ERα is a transcriptional factor allosterically regulated by ligand, which promotes gene transcription by recruiting coactivator proteins in a ligand-dependent manner (31). Because melatonin actions involve alterations on ER structure that could interfere with the association of factors required for ER activity, we analyzed whether melatonin affects the binding of the coactivator SRC-1a to ERα. GST pull-down experiments were performed by using ^{35}S-labeled SRC-1a and GST-ERα-(117–595) purified and immobilized on GSH-Sepharose as an affinity reagent (Fig. 7, *A* and *B*). As expected, the binding of SRC-1a to ERα was stimulated 3-fold in the presence of E_2, and the presence of the estrogenic antagonists 4-hydroxytamoxifen (OHT) and ICI blocked this association to ERα (28). By contrast, the E_2-dependent interaction of SRC-1a with ERα was unaffected by melatonin. Both melatonin and anti-estrogens failed to induce by themselves SRC-1a association to ERα. These results strongly suggest that the mechanism of melatonin action differs from those of anti-estrogens such as OHT or ICI, which exert inhibitory effects on ER activity by impairing coactivator recruitment.

Because melatonin induces conformational changes on ERα structure and this fact seems to have no consequences on the binding of coactivators, we postulate that CaM and coactivator binding to ER are independent phenomena. To check this, we determined whether forms of ERα unbound to CaM retain the ability to interact with coactivators. This was accomplished in another set of GST pull-down experiments using ^{35}S-labeled SRC-1a and the hybrid protein GST-ERα-(280–595), which is unable to bind CaM (18) immobilized on GST-Sepharose as affinity reagent (Fig. 7, *C* and *D*). Under these conditions the binding of SRC-1a to GST-ERα-(280–595) was induced by E_2, and once again the presence of melatonin had no effect on this interaction, whereas the presence of OHT impaired SRC-1a association. These results indicate that the binding of CaM to ERα is not a prerequisite for the recruitment of coactivators and that these are two independent mechanisms for the regulation of ERα activity.

Melatonin Selectively Prevents the Binding of E_2·ERα Complex to ERE in Vitro—We have investigated whether the selective inhibition of ERα-mediated transcription by melatonin is exerted at the level of DNA binding. To accomplish this, we

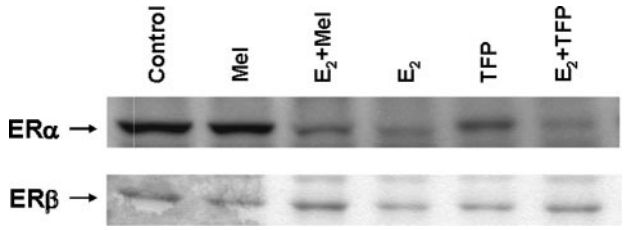

FIG. 6. **Effect of melatonin on estrogen receptor content.** MCF7 cells synchronized in 0.5% charcoal, dextran-treated fetal calf serum were stimulated for 24 h with vehicle (*Control*), 100 nM melatonin (*Mel*), 10 nM E_2, 20 μM trifluoperazine (*TFP*), or combinations as indicated. The relative amount of endogenous ERs present in whole cell extracts was analyzed by immunoblotting with antibodies directed against ERα or ERβ as indicated.

conducted electrophoretic mobility shift assays using nuclear extracts from HeLa cells transfected with ERα or ERβ to determine the effect of melatonin on the E_2-dependent binding of each ER isoform to ERE. ERα binding to ERE was increased 3-fold in the presence of 10 nM E_2 (Fig. 8, *lane 2*). This binding was 90% inhibited by the addition of 1 nM melatonin (Fig. 8, *lane 3*). In a similar way the binding of ERβ to ERE was also increased 2.5-fold in the presence of E_2 (Fig. 8, *lane 5*), but contrary to ERα the addition of melatonin stimulated the binding of ERβ to DNA (Fig. 8, *lane 6*). The specificity of the retarded band was demonstrated by competition with a 100-fold excess of unlabeled ERE (Fig. 8, *lane 7*).

Taken together, our results indicate that melatonin induces conformational changes in CaM that selectively prevent ERα-dependent transcription by destabilizing the binding of E_2·ERα·CaM complex to DNA either by modulating ER stability at protein level or by impairing coactivator binding to the receptor.

DISCUSSION

Melatonin is an indole hormone secreted by the pineal gland only during the night or, more exactly, in darkness. One of the proposed properties of melatonin is its role as an oncostatic agent on hormone-dependent tumors. It has also been described that melatonin exerts antiproliferative effects on MCF7 cells, which has become a useful model to study the anti-estrogenic effect of the pineal hormone (11, 32–35). In synchronized MCF7 cells, both estrogen-dependent transcription and proliferation are inhibited by co-treatment with melatonin. It has been shown that melatonin binds to calmodulin in a Ca^{2+}-dependent fashion, resulting in inhibition of calmodulin (16, 17). We have proposed (14) that calmodulin might be a potential candidate to mediate the anti-estrogenic effects of melatonin. In this regard, we have recently demonstrated that ERα but not ERβ interacts with calmodulin, and mutations in the postulated (36) ERα calmodulin binding site abolish this inter-

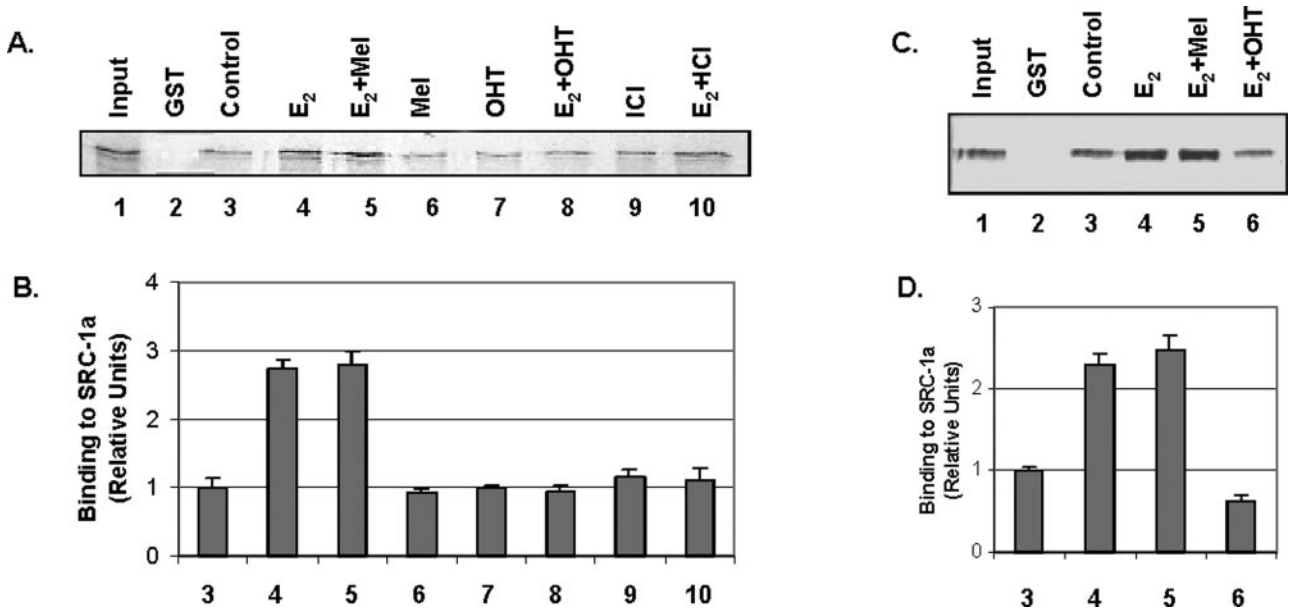

FIG. 7. **Effect of melatonin on E_2-dependent binding of the SRC-1a coactivator to ERα.** *A*, *in vitro* translated ^{35}S-labeled SRC-1a was incubated with GST-ERα-(117–595), purified, and immobilized on GSH-Sepharose in the absence of ligand (*C*) or in the presence of either 1 μM E_2, 1 μM OHT, 1 μM ICI 182,780 (*ICI*), or 1 nM melatonin (*Mel*) alone or combination as indicated. The *Input lane* represents 20% of the total amount of labeled SRC-1a used in the binding reactions (*lane 1*). *B*, quantification of the specific band was determined by densitometry. The results are expressed as relative units with respect to *C* and represent the mean ± S.D. of two independent experiments. *C*, effect of melatonin on E_2-dependent binding of the SRC-1a coactivator to CaM-unligated form of ERα. *In vitro* translated ^{35}S-labeled SRC-1a was incubated with the GST-ERα-(280–595), purified, and immobilized on GSH-Sepharose in the absence of ligand (*C*) or in the presence of 1 μM E_2, 1 μM OHT, and 1 nM melatonin as indicated. The *Input lane* represents 10% of the total amount of labeled SRC-1a used in the binding reactions (*lane 1*). *D*, quantification of the specific band was determined by densitometry. The results are expressed as relative units with respect to *C* and represent the mean ± S.D. of two independent experiments.

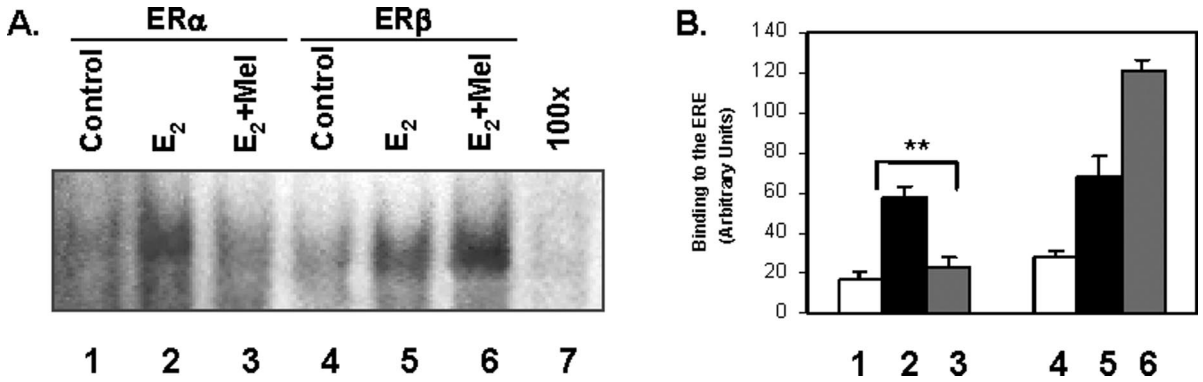

FIG. 8. **Effect of melatonin on estradiol-dependent binding of both ERα and ERβ to ERE *in vitro*.** *A*, nuclear extracts from HeLa cells expressing ERα or ERβ were assayed for ERE binding activity by EMSA in the absence (*Control*) or in the presence of 10 nM E_2 plus or minus 1 nM melatonin. In *lane 7*, the binding of ER to ERE was competed by a 100-fold excess of unlabeled ERE. *B*, quantification of the specific band was determined by densitometry. The results are expressed as arbitrary units and represent the mean ± S.D. of three independent experiments. **, significantly different; $p < 0.01$.

action (18). The mutant receptor is otherwise fully functional promoting E_2-dependent transcription. As a consequence of the interaction of the receptor with this calcium ligand protein, CaM antagonists act as specific inhibitors of ERα in a dose-dependent manner but show no inhibitory effect on ERβ- mediated transcription both in ERE- and AP1-driven promoters (18). Considering the observations mentioned above, we addressed the question of whether the inhibitory effects observed in MCF7 cells treated with melatonin were exerted via CaM. For this purpose we studied the effect of melatonin on E_2-dependent transactivation mediated by each receptor in transfected HeLa cells. We found that similarly to CaM antagonists, melatonin inhibits E_2·ERα-induced transcription at several ERE-driven promoters, whereas ERβ activation is not inhibited (or even enhanced at certain promoters) by treatment with the hormone. Importantly, the ERα (K302G,K303G) mutant, which does not interact with CaM, is not inhibited by melatonin and behaves in a similar fashion to that of the ERβ receptor. This result strongly suggests that the effect of the hormone is directly exerted through the calmodulin bound to ERα and not through indirect pathways. All the data mentioned above indicate that melatonin is a selective modulator of estrogen receptors. This observation is extremely important since more than 60% of breast cancers show overexpression of ERα, and the ratio ERα/ERβ increases during breast and ovarian tumor progression (37). Therefore, melatonin, as we have previously suggested for CaM antagonists, might have the potential to act as an ERα inhibitor with antitumor effects on advanced breast, ovarian, and colon cancer.

We have attempted to determine if the inhibitory effects observed in MCF7 cells treated with melatonin are associated with the high ERα/ERβ ratio present in these cells as we have reported for CaM antagonists (18). Indeed, in MCF7 cells expressing ectopic ERβ, the inhibitory effects of melatonin and

CaM antagonists were abolished. These results imply that the sensitivity to melatonin correlates with a high ERα/ERβ ratio.

Because we had previously found that CaM antagonists also inhibit ERα-dependent AP1 transcriptional activity (18) we tested the ability of melatonin to inhibit ERα-mediated transcription in AP1-driven promoters. As expected, melatonin significantly inhibited AP1/ERα activation by E_2. Therefore, melatonin also acts as a regulator of the ERα-CaM/AP1 pathway. CaM inhibitors and tamoxifen have been reported to show synergistic inhibitory effects (33). Thus, we propose that melatonin could be a valuable tool to block the mitogenic activity of ERα in both anti-estrogen-responsive and anti-estrogen-resistant breast cancer cells.

We have previously reported that melatonin blocks the binding of E_2·ER complex to ERE *in vitro*, but other methoxy- and hydroxyindoles were not effective in doing so. Therefore, we predicted that these compounds would have neither effect on E_2-dependent transactivation nor ability to bind dansyl-CaM. The effect of melatonin is indeed, specific, since none of the compounds tested inhibited E_2-dependent transcription or modified the fluorescence of dansyl-CaM.

The next question we addressed was whether melatonin has any effect on the stability of ERα, as it has been described for other calmodulin antagonists (30). MCF7 cells treated with CaM antagonist TFP show a 50% reduction in the ER levels, and nearly all the receptor disappeared when CGS9343B was used. These compounds did not significantly modify the level of ER mRNA. It has been, therefore, proposed that calmodulin stabilizes the receptor against proteolysis (30). The same authors suggested that the inhibitory effect of W7 on ERα-mediated transcription that we have previously reported (18) might be due to the reduction in ER-protein levels induced by CaM antagonists (38, 39). Treatment with melatonin did not alter the amount of ERα present in MCF7 cells, whereas cells treated with TFP did show a reduction in the ER levels similar to that previously reported (37). Therefore, the possibility that melatonin inhibits ERα transcription as a consequence of degradation of the receptor can be excluded. Notably, the levels of ERβ remain unaffected in all the conditions assayed.

We next investigated melatonin to determine if it might interfere with the association of factors required for ERα activity. Anti-estrogens induce a conformational change in the receptor different to that of E_2 in such way that corepressors and not coactivators bind to the receptor in the presence of the tamoxifen (40). We, therefore, considered the possibility that melatonin might have a similar effect and that the conformational change induced by melatonin on CaM might imply another change on ERα structure in such way that coactivator association to the receptor would be impaired. We have analyzed the binding of the coactivator SRC-1a to ERα. We found that, as previously reported (41, 42), the binding of SRC-1a to ERα was stimulated by E_2, whereas the presence of the estrogenic antagonists OHT and ICI blocked this association. On the other hand, E_2-dependent interaction of SRC-1a with ERα was unaffected by melatonin, indicating that this is not the mechanism by which melatonin exerts its inhibitory effect on ERα-mediated transcription. We have also shown that CaM and coactivator binding to ERα are independent phenomena, since a GST fusion with a truncated ERα unable to interact with CaM recruits SRC-1a, and this association is enhanced by estradiol and inhibited by OHT and ICI. In other words, our results strongly suggest that CaM binding to ERα is not a necessary event for coactivator recruitment. This idea is further supported by the fact that the mutant ERα (K302G,K303G), which does not bind CaM, mediates transcription in a similar way to that of wild-type ERα, although treatment with melatonin does not inhibit its actions. How then does melatonin inhibit transcription? We have investigated whether the differential action of melatonin on E_2-dependent transcription by ERα and ERβ was exerted on binding of ER to DNA. We performed electrophoretic mobility shift assays using nuclear extracts from HeLa cells expressing either ERα or ERβ to determine the effect of melatonin on the E_2-dependent binding of each receptor isoform to ERE. The binding of ERα to DNA was increased in the presence of estradiol and inhibited by melatonin. On the other hand, melatonin further enhanced the binding of ERβ to DNA, which might explain why at certain promoters co-treatment with E_2 and melatonin enhanced ERβ transcriptional activity.

As mentioned above, the ratio ERβ/ERα, which is high in normal tissues, decreases during breast and ovarian tumor progression (43) and in both male and female malignant transformation of the colon (44, 45). Notably a large scale study conducted on long time night-shift workers indicates that those working night shifts at least three nights per month for 15 years or longer have a moderate but significant increase in the risk of developing breast and colon cancer (6, 46). Because the levels of ERβ are reduced during tumor progression in most colon cancers, melatonin might be important for prevention of the breast and colon malignancies acting through ERα. Our results favor the concept that melatonin and CaM antagonists could be of therapeutic importance in tumors with high ERα/ERβ ratio, that is, in advanced tumors. In addition, CaM antagonists alone or in combination with anti-estrogens have been reported to decrease the viability and induce apoptosis of breast cancer cells (47–49). Melatonin decreased toxicity and increased efficacy of cancer chemotherapy in metastatic patients with poor clinical status (50).

In summary, the reported data and the results presented in this work strongly suggest that melatonin or melatonin derivatives with no toxicity and higher efficacy than the pineal hormone may have the potential to act as inhibitory agents of ERα with anti-tumor effects on advanced breast, ovarian, and colon tumors. Therefore, clinical studies on the possible therapeutic value of melatonin on these cancers should be performed in the future.

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Mechanisms of Signal Transduction:

Melatonin, an Endogenous-specific Inhibitor of Estrogen Receptor α via Calmodulin

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