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Melatonin inhibits both ERα activation and breast cancer cell proliferation induced by a metalloestrogen, cadmium

Abstract: Cadmium (Cd) is a heavy metal affecting human health both through environmental and occupational exposure. There is evidence that Cd accumulates in several organs and is carcinogenic to humans. In vivo, Cd mimics the effect of estrogens in the uterus and mammary gland. In estrogen-responsive breast cancer cell lines, Cd stimulates proliferation and can also activate the estrogen receptor independent of estradiol. The ability of this metalloestrogen to increase gene expression in MCF7 cells is blocked by anti-estrogens suggesting that the activity of these compounds is mediated by ERα. The aims of this work were to test whether melatonin inhibits Cd-induced proliferation in MCF7 cells, and also to study whether melatonin specifically inhibits Cd-induced ERα transactivation. We show that melatonin prevents the Cd-induced growth of synchronized MCF7 breast cancer cells. In transient transfection experiments, we prove that both ERα- and ERβ-mediated transcription are stimulated by Cd. Melatonin is a specific inhibitor of Cd-induced ERα-mediated transcription in both estrogen response elements (ERE)- and AP1-containing promoters, whereas ERβ-mediated transcription is not inhibited by the pineal indole. Moreover, the mutant ERα-(K302G, K303G), unable to bind calmodulin, is activated by Cd but becomes insensitive to melatonin treatment. These results proved that melatonin inhibits MCF7 cell growth induced by Cd and abolishes the stimulatory effect of the heavy metal in cells expressing ERα at both ERE-luc and AP1-luc sites. We can infer from these experiments that melatonin regulates Cd-induced transcription in both ERE- and AP1 pathways. These results also reinforce the hypothesis of the anti-estrogenic properties of melatonin as a valuable tool in breast cancer therapies.

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Introduction

Cadmium (Cd) is an environmental pollutant widely used in industry and which is also present in tobacco smoke. Activities such as smelting, fuel combustion, smoking, or Ni-Cd battery manufacturing are the main sources of human exposure to this heavy metal which, because of its long biological half-life, estimated to be between 15 and 20 yr [1], accumulates in the body.

Cadmium has been classified as a human carcinogen by the International Agency for Research on Cancer [2]. Epidemiological studies have identified lung, prostate, kidney and stomach as targets for Cd-induced tumorigenesis [2]. In rats, Cd can induce prostate and testicular tumors [3]. The mechanisms of Cd carcinogenesis are not well understood. Cd is cytotoxic and induces radical-dependent DNA-damage at concentrations ranging from 0.1 to 10 mM [4], whereas at lower doses (1–100 μM) it decreases DNA replication and repair [5], and up-regulates the expression of proto-oncogenes such as c-fos, c-jun and c-myc [6]. It has also been described that Cd perturbs the folding of p53 and impairs p53 induction by DNA-damaging agents inactivating the wild-type protein [7]. Cd also damages the E-Cadherin-dependent junctions between cells, a fact that could represent an important step in tumor initiation and promotion [8, 9].

There is increasing evidence relating Cd and breast cancer. It has been reported that Cd has a potent estrogen-like activity in vivo. In rats, the exposure to this heavy metal increases uterine weight, induces the expression of progesterone receptor (PgR), increases the proliferation of the endometrium, and promotes growth and development of the mammary glands increasing the formation of side branches and alveolar buds and well as the production of casein and whey acidic protein [10].

In breast cancer cells, Cd acts like steroidal estrogens which induce MCF7 proliferation, probably by forming a high-affinity complex with the hormone binding domain of the estrogen receptor alpha (ERα) [11–12]. MCF7 cells treated with Cd showed decreased levels of ER protein and ER mRNA [11]. On the contrary, Cd treatment increases PgR receptor and cathepsin D mRNA levels. The heavy

metal also stimulates estrogen response elements (ERE) in transient transfection experiments [11].

Melatonin, the major secretory product of the pineal gland, has oncostatic and anti-proliferative effects on endocrine-responsive neoplasms, especially in those concerning the mammary gland [13, 14]. The most common conclusion in animal models of tumorigenesis is that either experimental manipulations that activate the pineal gland or the administration of melatonin is what reduces the incidence and development of chemically induced mammary tumors, whereas pinealectomy usually stimulates breast cancer growth [15–17]. Different mechanisms have been proposed to explain how melatonin could reduce the development of mammary tumors. Studies using MCF7 human breast cancer cells demonstrate that physiological concentrations of melatonin (1 nM to 1 pM) exert a direct anti-proliferative effect on estradiol (E_2)-induced proliferation of these cells [18, 19] and reduce their invasiveness, causing a decrease in cell attachment and cell motility, probably by interacting with estrogen-mediated mechanisms [20]. It has been demonstrated that melatonin inhibits MCF7 cell proliferation by inducing an arrest of cell cycle, dependent on an increased expression of p21WAF1 protein, which is mediated by the p53 pathway [21]. Melatonin acts as an anti-estrogen by preventing the E_2-dependent transcriptional activation in MCF7 cells through destabilization of the E_2-ER complex from binding to DNA [22]. Calmodulin (CaM) was proposed as a potential candidate for mediating the anti-estrogenic effects of melatonin [23]. Indeed, it has been recently reported that melatonin is a specific inhibitor of E_2-induced ERα-mediated transcription in both ERE- and AP1-containing promoters, whereas ERβ-mediated transactivation is not affected. Moreover, the mutant ERα (K302G, K303G), unable to bind CaM, normally transactivates in response to E_2 treatment, but becomes insensitive to melatonin [24]. 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 [24].

The aims of the present work were to test whether melatonin inhibits Cd-induced proliferation in MCF7 cells, and also to study whether melatonin specifically inhibits Cd-induced ERα transactivation. The results obtained proved that, indeed, melatonin inhibits MCF7 cell growth induced by Cd and the stimulatory effect of the heavy metal in cells expressing ERα at both ERE-luc and AP1-luc sites was abolished by treatment with melatonin. We can infer from these experiments that melatonin regulates Cd-induced transcription in both ERE- and AP1 pathways.

Materials and methods

Materials

Melatonin, 17β-estradiol (E_2), Epidermal Growth Factor (EGF), cadmium chloride, 4-hydroxytamoxifen, and other chemicals were purchased from Sigma-Aldrich (Madrid, Spain).

Plasmids

The expression vector pcDNA-ERα has been previously described [23]. pCMX-mERβ were kindly provided by Dr V. Giguére from the R.W. Johnson Pharmaceutical Research Institute, Don Mills, Ontario, Canada. 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, USA. ΔColl-73 was kindly provided by Dr A. Aranda from Instituto de Investigaciones Biomédicas 'Alberto Sols'CSIC Madrid, Spain. pRL-TK (Promega Corp., Madison, WI, USA) was also used in this work.

Cell proliferation assays

MCF7 cells were seeded in 96-multiwell plates at a density of 6000 per well and incubated for 48 hr at 37°C in Dulbecco's Modified Eagle's Medium (DMEM) with 5% of charcoal/dextran-treated fetal calf serum (sFCS). At 60–80% confluence, the media were replaced by fresh ones containing 5% sFCS as well as $CdCl_2$ (1 μM), 17β-estradiol (10 nM), melatonin (1 nM) or Cd plus melatonin at the same concentrations as above. Cells were cultured for 5 days. Medium was renewed 72 hr after the beginning of treatment. Cell proliferation was measured by the MTT [3(4,5dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Molecular Probes Inc. Eugene, OR, USA) method reading absorbance at 570 nm in a microplate reader.

Transient transfection assays

HeLa cells were propagated as previously described [23]. Before transfection, HeLa cells were seeded in 12-well plates and incubated 12–18 hr at 37°C. Then, cells were transferred to phenol-red free DMEM containing 0.5% sFCS and maintained for 3 days. At 60–80% confluency, cells were transfected with 0.75 μg of ERE-driven or AP1-driven reporter plasmids, 0.1 μg of ER expression vector and 75 ng of an internal control *Renilla* luciferase plasmid, pRL-TK (Promega Corp., Fitchburg, WI, USA) using FuGENE 6 Transfection Reagent from Roche Molecular Biochemicals (Mannheim, Germany) following the manufacturer's protocols. After 18–24 hr, medium was renewed and cells were stimulated during 24 hr with different chemicals, as indicated.

Luciferase was assayed with the Dual Luciferase System (Promega Corp.). Luciferase activities were normalized to *Renilla* luciferase activity, in order to correct for differences in transfection efficiency. The results represent the mean ± S.D. of three independent experiments performed at least in duplicates.

MCF-7 cells were propagated in RPMI 1640 medium containing 25 mM HEPES/NaOH pH 7.3 and synchronized cells were transfected as above.

Statistical analysis

The data on luciferase activity and cell proliferation are expressed as the mean ± standard errors of the mean (S.E.M.). Statistical differences between groups were

processed by one way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test. Results were considered as statistically significant at $P < 0.05$.

Results

To investigate whether or not melatonin inhibits the growth of MCF-7 cells induced by Cd, cells were cultured in DMEM with 5% of estrogen-depleted fetal bovine serum (sFCS). The addition of 1 μM $CdCl_2$ into media stimulates cell proliferation (Fig. 1, bar 2). The stimulation levels on MCF7 proliferation reached with this concentration of Cd was similar to that obtained with 10 nM E_2 (Fig. 1, bar 3). This proliferative effect of Cd was significantly inhibited by 1 nM melatonin (Fig. 1, bar 5).

To assess whether Cd stimulates both ERα- and ERβ-mediated transcription, and whether melatonin is able to specifically inhibit ERα transactivation mediated by the heavy metal (as has already been described for E_2), HeLa cells were transiently transfected with either ERα or ERβ expression vectors along with a 3x-ERE-TATA-Luc plasmid. Both ERα (Fig. 2A, bar 4) and ERβ (Fig. 2B, bar 4) are able to effectively mediate Cd transcriptional activation. Melatonin (100 nM) inhibited ERα-mediated transactivation by 45–60% (Fig. 2A, bar 5), whereas ERβ-mediated transcription was not affected by this concentration of melatonin (Fig. 2A, bar 5). Therefore, we can conclude from this set of experiments that melatonin is a specific inhibitor of ERα-mediated transcription induced by Cd in the same way that the pineal hormone inhibits ERα-mediated transcription induced by estradiol. The mutant ERα(K302G, K303G), which is unable to bind to CaM, was stimulated by 1 μM Cd (Fig. 2C, lane 4). Melatonin was unable to counteract Cd transcriptional activation mediated by ERα (K302G, K303G) (Fig. 2C, bar 5).

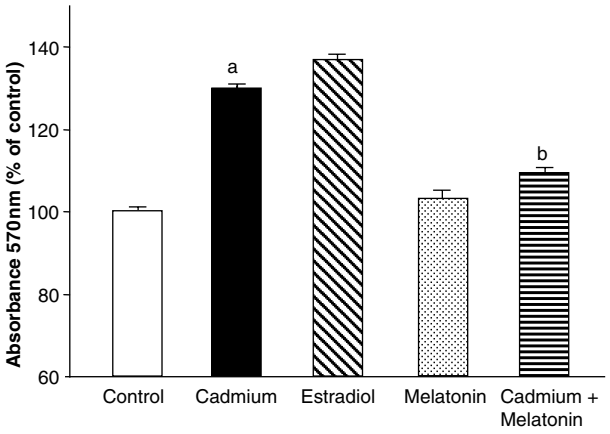

Fig. 1. Cadmium (Cd) induced cell proliferation. Inhibitory effect of melatonin. MCF7 cells were seeded in 96-multiwell plates at a density of 6000 per well and incubated for 48 hr at 37°C in DMEM with 5% sFCS. At 60–80% confluence, the media were replaced by fresh ones containing 5% cFCS as well as $CdCl_2$ (1 μM), 17β-estradiol (10 nM), melatonin (1 nM) or Cd (1 μM) plus melatonin (1 nM). Cells were cultured for 5 days and cell proliferation was measured by the MTT method. a, $P < 0.001$ versus control; b, $P < 0.001$ versus Cd.

To date, no data have been available indicating whether or not Cd can activate ERα/AP1 and/or ERβ/AP1 pathways. To address this question, HeLa cells were transfected with either ERα or ERβ along with the reporter plasmid Δ coll 73-Luc (containing an AP1 binding site). We found that Cd synergizes with EGF to significantly enhance AP1 activity in ERα-transfected cells (Fig. 3A, bar 3) proving that treatment with Cd can stimulate ERα/AP1 pathways. Our data show that in ERα/AP1 Cd (1 μM) activation is significantly higher than that obtained with 1 nM E_2 (Fig. 3A, compare bar 3 and 5). Very importantly, the synergistic effect of EGF and Cd in cells expressing ERα was sensitive to melatonin, this inhibition being statistically significant (Fig. 3A, lane 4). Interestingly, Cd does not diminish AP1 activity in ERβ-transfected cells (Fig. 3B, bar 3) as E_2 does (Fig. 3B, bar 5). Melatonin does not have any inhibitory effect on AP1 activity mediated by Cd through ERβ (Fig. 3B, bar 4). From these experiments we can infer that melatonin regulates Cd-ERα-mediated transcription not only in ERE-dependent pathways but also in AP1 pathways, and importantly, the pineal hormone inhibits the ERα/AP1 pathway independently of the ERα activator (E_2 or Cd), whereas no effect was observed in cells expressing ERβ.

Discussion

Cadmium is a heavy metal that is dispersed throughout the modern environment and that accumulates in the body. Human exposure to Cd occurs primarily through dietary sources, cigarette smoking and drinking water [25–28]. Prolonged exposure to Cd has been linked to multiple toxic effects in both humans and animals and this heavy metal has been classified as a human carcinogen by the International Agency for Research on Cancer.

Breast cancer is the leading cause of death in woman between the ages of 35 and 45 [29]. Because the ER is a mediator of growth, molecules that bind and activate this receptor can potentially increase the risk of breast cancer. Thus, there is strong evidence that Cd is a potent nonsteroidal estrogen in vivo (metalloestrogen). Cd has been reported to mimic the effects of E_2 in the estrogen responsive cell line MCF7 stimulating cell proliferation [11] as a result of its ability to form a high affinity complex with the hormone binding of the ER [12]. As E_2 also stimulates AP1 [30, 31], our aim was to test the effects of Cd in both ERE- and AP1 pathways.

Melatonin is an indole hormone secreted by the pineal gland only during the night or, more exactly, in darkness. Among the properties of melatonin, we can underline its role as an oncostatic agent on hormone-dependent tumors [13, 18, 20]. It has also been described that melatonin exerts anti-proliferative effects on MCF7 cells, which has become a useful model to study the anti-estrogenic effect of the pineal hormone [32–34]. In synchronized MCF7 cells E_2-induced proliferation is inhibited by co-treatment with melatonin. As treatment with Cd also stimulates growth of this cell line, we decide to test the ability of melatonin to act as an anti-proliferative agent; our results show that melatonin can block the proliferation of MCF7 cells triggered by Cd

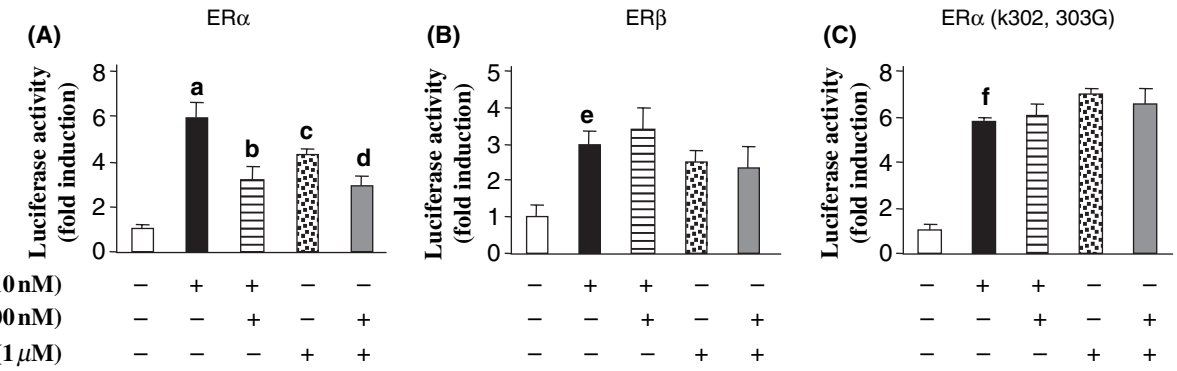

Fig. 2. Differential effect of melatonin on the transactivation properties of (A) ERα, (B) ERβ and (C) ERα (K302G, K303G). HeLa cells were transfected with 0.1 μg of ERα, ERβ or ERα (K302G, K303G) expression vectors, 50 ng of the internal control plasmid pRL-TK and 0.75 μg of 3xERELuc (ERE-driven reporter plasmid). After 18–24 hr, medium was renewed and cells were stimulated for 24 hr with 10 nM E_2, 1 μM cadmium (Cd) and 100 nM melatonin 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 mean ± S.D. of three independent experiments performed in duplicates. a, $P < 0.001$ versus control; b, $P < 0.001$ versus E_2; c, $P < 0.001$ versus control; d, $P < 0.001$ versus Cd; e, $P < 0.001$ versus control; f, $P < 0.001$ versus control.

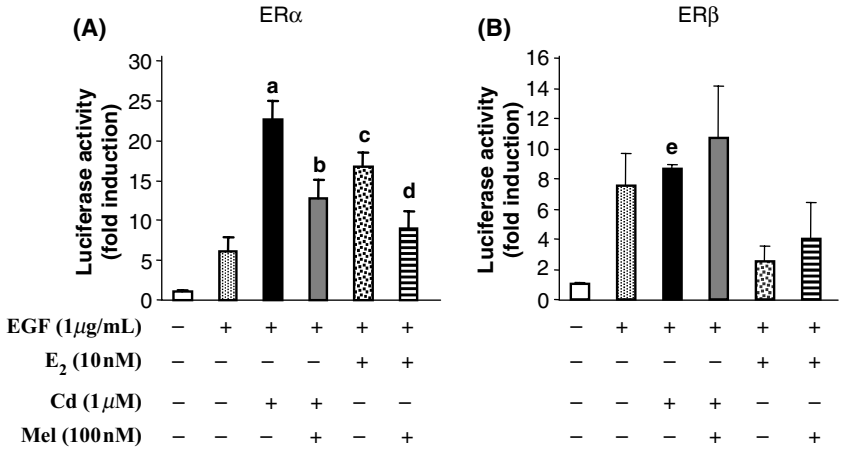

Fig. 3. Effect of melatonin on cadmium (Cd)-dependent transactivation at an AP1 element. (A) ERα and (B) ERβ HeLa cells were transfected with 0.1 μg of the ERα expression vector, 50 ng of internal control plasmid pRL-TK and 0.75 μg of the AP1-containing reporter plasmid (Δ coll. 73-Luc). Cultures were stimulated for 48 hr with 1 μg/mL EGF, 10 nM E_2, 1 μM Cl_2Cd and 100 nM of melatonin as indicated. The data are reported as fold-induction relative to untreated cells, which were arbitrarily assigned as 1. The bars represent mean ± S.D. of three independent experiments run in duplicates; a, $P < 0.001$ versus EGF; b, $P < 0.001$ versus EGF plus Cd; c, $P < 0.001$ versus EGF plus Cd; d, $P < 0.001$ versus EGF plus estradiol; e $P < 0.001$ versus estradiol plus EGF.

thus confirming, one more time, the anti-estrogenic nature of melatonin actions [35].

Both ERα and ERβ bind the same DNA sequence and activate the transcription of genes regulated by ERE. However, only ERα, but not ERβ, which has a CaM binding site, interacts with CaM and is specifically inhibited by both CaM antagonists and melatonin [23, 24]. Therefore, one of the aims of this work was first to test whether or not both ERα and ERβ are stimulated by Cd, and secondly, whether melatonin specifically inhibited ERα but showed no inhibitory effect over ERβ-mediated transcription. We found that both ERα and ERβ are significantly activated by Cd and that Cd-induced transcription mediated by ERβ reaches lower levels than that of ERα, as has been previously described for E_2. Cd-induced transcription through ERα is inhibited by melatonin at ERE-driven promoters, as previously shown for E_2, whereas ERβ activation is not affected by treatment with the pineal hormone. Importantly, the ERα (K302G, K303G) mutant, which does not interact with CaM, is stimulated by Cd but is not inhibited by melatonin. Therefore, melatonin acts as an ERα specific inhibitor with independence of the agent used for cell growth stimulation, either as previously shown for E_2 or Cd.

We also addressed the effect of Cd in AP1 sites. It has been reported that exposure of cells to Cd-induced significant activation of AP1 and all three members of the MAP kinase family in mouse epidermal JB6 cells. The induction of AP1 activity by Cd appears to involve activation of Erks, since the induction of AP1 activity by Cd was blocked by pretreatment of cells with PD98058 [36]. In primary rat hepatocytes, it has been observed that Cd, through the

generation of reactive oxygen species and prior to significant cellular damage, activates the stress activated signal protein JNK, regulates c-jun expression, and promotes the binding of a redox sensitive transcription factor AP1 [37].

It has been documented that ERα and ERβ signal in opposite ways when complexed with the natural hormone E_2 from an AP1 site: with ERα, 17β-estradiol activates transcription, whereas with ERβ, 17β-estradiol inhibits transcription [38]. The effect of many environmental estrogenic chemicals on AP1 has been tested with either ERα or ERβ in NIH 3T3 cells. Compounds such as bisphenol A or t-methylbutylphenol activate only ERα but not ERβ-dependent AP1 transcriptional activity [39].

For all the reasons mentioned above, we tested the ability of Cd to modulate both ERα- and ERβ-dependent AP1 transactivation. We found that Cd significantly stimulates AP1 activity in ERα-transfected cells. Interestingly, whereas 17β-estradiol inhibits transcription on AP1 through ERβ, Cd neither stimulates nor inhibits transcription on this system.

Because it has been previously described that CaM antagonists and melatonin also inhibit ERα-dependent AP1 transcriptional activity [23, 24] we tested the ability of melatonin to inhibit ERα-mediated transcription in AP1-driven promoters. We found that melatonin significantly inhibited ERα-mediated transactivation in AP1 sites; therefore, melatonin also acts as a regulator of the ERα-CaM/AP1 pathway when this pathway is stimulated by Cd.

In summary, as chemicals such as Cd mimic the effect of estrogens, the high incidence of breast cancer among women working in the chemical industry could be explained because of the estrogenic properties of this heavy metal. The protective effects of melatonin on Cd-induced breast cancer cell proliferation, now demonstrated, point to a possible role of this indolamine as a preventive agent for Cd environmental or occupational contamination.

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