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ORIGINAL ARTICLE

Melatonin inhibits the proliferation of breast cancer cells induced by bisphenol A via targeting estrogen receptor-related pathways

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Keywords
Bisphenol A; breast neoplasms; estrogen response element; melatonin; steroid receptor coactivator.

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Abstract

Background: Background: Bisphenol A (BPA) is an estrogen-like chemical widely contained in daily supplies. There is evidence that environmental exposure to BPA could contribute to the development of hormone-related cancers. As is reported in numerous studies, melatonin, an endogenous hormone secreted by the pineal gland, could markedly inhibit estrogen-induced proliferation of breast cancer (BC) cells. In this study, we intended to reveal the effects of melatonin on BPA-induced proliferation of estrogen receptor-positive BC cells.
Methods: Methods: We used methyl thiazolyl tetrazolium, luciferase reporter gene and western blotting assays to testify the effect of melatonin on BPA-mediated proliferation of MCF-7 and T47D cells.
Results: Methyl thiazolyl tetrazolium and colony formation assays showed that melatonin could significantly abolish BPA-elevated cell proliferation. Meanwhile, BPA-upregulated phosphorylation of ERK and AKT was decreased by melatonin treatment. Mechanistically, we found that BPA was capable of upregulating the protein levels of steroid receptor coactivators (SRC-1, SRC-3), as well as promoting the estrogen response element activity. However, the addition of melatonin could remarkably block the elevation of steroid receptor coactivators expression and estrogen response element activity triggered by BPA.
Conclusion: Conclusions: Therefore, these results demonstrated that melatonin could abrogate BPA-induced proliferation of BC cells. Therapeutically, melatonin could be regarded as a potential medication for BPA-associated BC.

Introduction

Bisphenol A (BPA) is a carbon-based synthetic compound that has been widely used in many daily supplies, including dental sealants, food packaging, and plastics polycarbonate polyvinyl chloride.[1,2] Under heat, acidic and basic conditions, or constant use, these products could release BPA to the environment.[3,4] Exposure to BPA would jeopardize the human immune system and the female reproductive system.[1,5] Breast cancer (BC) is a malignant carcinoma that severely threatens women's health. Many studies have reported that BPA shows estrogen-like properties and has a link with BC development.[6,7] Estrogen and estrogen-like compounds normally interact with estrogen receptor (ER), and then modulate BC progression through two mechanisms: (i) directly bind with estrogen response element (ERE) to regulate gene expression; and (ii) work through a rapid non-genomic mechanism including activating the MAPK and PI3K/AKT pathways.[8,9] As an estrogen-like chemical, BPA could imitate estrogen to interact with ER, resulting in abnormal cell proliferation, migration, and apoptosis.[6,10]

Melatonin, a secretion from the pineal gland, is an important endogenous hormone in regulating circadian rhythm.[11] The synthesis and secretion of melatonin could be disrupted by exposure to light at night.[12] However, with the compact modern lifestyle, more and more people are becoming sleep deprived, which leads to disorder of melatonin's serum level. Several studies have substantiated that disruption of the normal circadian rhythm could lead to a higher risk of hormone-dependent cancer occurrence including BC.[11,13,14] Multiple in vivo and *in vitro* studies have demonstrated the effects of melatonin on reducing the incidence and growth rate of BC.[15,16] Further studies show that melatonin could effectively inhibit cell viability and proliferation, and induce apoptosis in ER-positive (ER^+) breast tumors.[17] However, whether melatonin could affect BPA-induced BC cell proliferation remains unknown.

To explore the effect of melatonin on BPA-mediated survival and proliferation of BC cells, we used MTT, colony formation, and western blotting assays in this study. We found for the first time that melatonin could inhibit BPA-elevated proliferation of MCF-7 and T47D cells, through suppressing ERE activity via declined expression of steroid receptor coactivators (SRCs), and downregulating AKT and ERK phosphorylation.

Methods

Materials

We purchased bisphenol A, 17β-estradiol (E2) and melatonin from Sigma-Aldrich (St. Louis, MO, USA). BPA and melatonin were dissolved in dimethyl sulfoxide as stock solutions, and 17β-estradiol was dissolved in ethyl alcohol. These three chemicals were reserved at −20°C. Goat monoclonal antibody against MT1 was purchased from Santa Cruz (Dallas, TX, USA). Rabbit primary monoclonal antibodies against SRCs, AKT, ERK1/2, phospho-AKT^{Ser473}, phospho-ERK1/2, p21, GAPDH, and anti-goat and anti-rabbit immunoglobulin G horseradish peroxidase-linked secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

Cell culture

MCF-7 and T47D cell lines were acquired from the American Type Culture Collection (ATCC, Rockville, MD, USA), and cultivated in 10% fetal bovine serum (FBS) supplemented Dulbecco's modified Eagle's medium (DMEM) or RPMI 1640 media (Gibco, Rockville, MD, USA) containing penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37°C with 5% CO_2.

Plasmids

The pERE-E1b-luc reporter plasmid, which contains the vitellogenin ERE, and pCMV-[beta]-galactosidase (pCMV-[beta]-gal) were kindly provided by C. Smith (Baylor College of Medicine).

MTT assay

Cell proliferation ability was determined by MTT assay. MCF-7 and T47D cells were seeded 3000 cells per well in 96-well plates with five replicates. The cells were incubated for 24 hours to form a monoplayer. Then, DMEM containing no FBS and no phenol-red was used to substitute for the culture media to starve for 24 hours. Indicated concentrations of BPA (100 nM), E2 (10 nM) or melatonin (1 nM) were added for another four days after starvation. The media were changed every 48 hours during the treatment. Then 10 μL MTT was added to each well for four hours' incubation. Later, the media were discarded and 150 μL dimethyl sulfoxide was added in each well to solve formazan. The absorption values were determined at OD_{490nm} by use of an absorbance reader (Enspire 2300 multimode reader; Perkin Elmer, Hopkinton, MA, USA).

Colony formation assay

A total of 500 cells for each well were seeded in 12-well plates containing DMEM with 10% FBS. The media were replaced by FBS and phenol red-free DMEM with the indicated dose of BPA, E2, and/or melatonin 48 hours later. Cells were

maintained in the incubator for 15 days. Distinguishable colonies were stained by crystal violet and calculated.

Protein preparation and western blotting assay

Expression of signaling pathway proteins were quantified by western blotting. We planted indicated cells at a density of 3×10^5 cells for each well. The next day, the media were replaced by DMEM with no phenol red and FBS to starve for 24 hours. Then, indicated BPA and melatonin were added in for another 48 hours. The protein extraction and sodium dodecyl sulfate-polyacrylamide gel electrophoresis were carried out as described previously.[18]

Transient transfection assay

Cells were seeded in 12-well plates and allowed to grow for 24 hours at 37°C. Later, cultural media were substituted by phenol red-free DMEM containing no FBS. After 12 hours starving, we transfected the cells with 1 μg of pERE-E1b-luc plasmid and pCMV-[beta]-gal packaged by Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA) as the manufacture's protocols instructed. Twelve hours later, media were renewed by which containing BPA or melatonin as indicated in previous experiment assays.

Luciferase reporter gene assay

To implement luciferase assay, we planted MCF-7 and T47D cells in 12-well plates, and then transfected them with pERE-E1b-luc reporter plasmid and pCMV-[beta]-gal (as control). After transfection for 12 hours, the media were replaced by DMEM with no FBS and no phenol red to starve for 12 hours. Then cells were treated with dimethyl sulfoxide, BPA, E2, and/or melatonin for 24 hours. The reporter gene activity was detected by a Luciferase Reporter Assay Kit (K801-200; BioVision, Mountain View, CA, USA) according to the manufacturer's specification. The fluorescence signal was measured using Enspire 2300 multimode reader (Perkin Elmer). The assay was processed in triplicate and at least three independent assays were carried out.

Statistical analysis

Student's *t*-test or one-way ANOVA were used to process the results in this study by SPSS13.0 software (SPSS, Chicago, IL, USA), among which the *P*-value <0.05 was regarded as significant.

Results

Melatonin could block the survival and proliferation of ER$^+$ BC cells induced by BPA

To investigate whether melatonin could abolish the survival and proliferation of ER$^+$ cell lines induced by BPA, we used DMEM media with phenol red-free and FBS to perform MTT assay. As shown in Figure 1a, the administration of 100 nM BPA could enhance the survival ratio of MCF-7 and T47D cells, which was similar to the effect reached by 10 nM E2. Another finding worth noting was that this rise could be significantly inhibited by the addition of 1 nM melatonin (Fig 1a). We also performed colony formation assay to testify the effect of melatonin on MCF-7 and T47D cell survival under BPA treatment. Cells exposed to BPA were able to form larger and more colonies, and melatonin could reverse this change (Fig 1b,c). Thus, we conclude that melatonin could block the survival and proliferation of ER$^+$ BC cell lines induced by BPA.

Melatonin is able to modulate the levels of ER-related key proteins under treatment of BPA

To further investigate the mechanism by which melatonin inhibits BPA-induced BC cell proliferation, we detected the levels of several proteins reported to be involved in ER-related cell proliferation. Phosphorylation of ERK and AKT were both obviously elevated when treated by BPA in MCF-7 and T47D cells (Fig 2a,b). However, melatonin could significantly abolish the upregulation of phosphorylated ERK and AKT mediated by BPA. Meanwhile, the effect of BPA on p21 could also be abrogated under melatonin treatment (Fig 2a,b). To explore whether the impacts of melatonin on ER$^+$ cells are related to melatonin receptor 1 (MT1), we detected the protein level of MT1.The result showed an upregulation of MT1 under treatment of melatonin, indicating that MT1 might be connected with melatonin-mediated abrogation of BC cell proliferation.

Melatonin is capable of inhibiting BPA-elevated SRC-1 and SRC-3

It was reported that BPA induced cell proliferation via its estrogen-like property.[6] Based on this property, we next investigated whether melatonin treatment affected BPA-induced proliferation by changing the expression of ER coactivators, SRC-1 and SRC-3. As shown in Figure 3, the upregulation of SRCs induced by BPA could be counteracted by addition of melatonin.

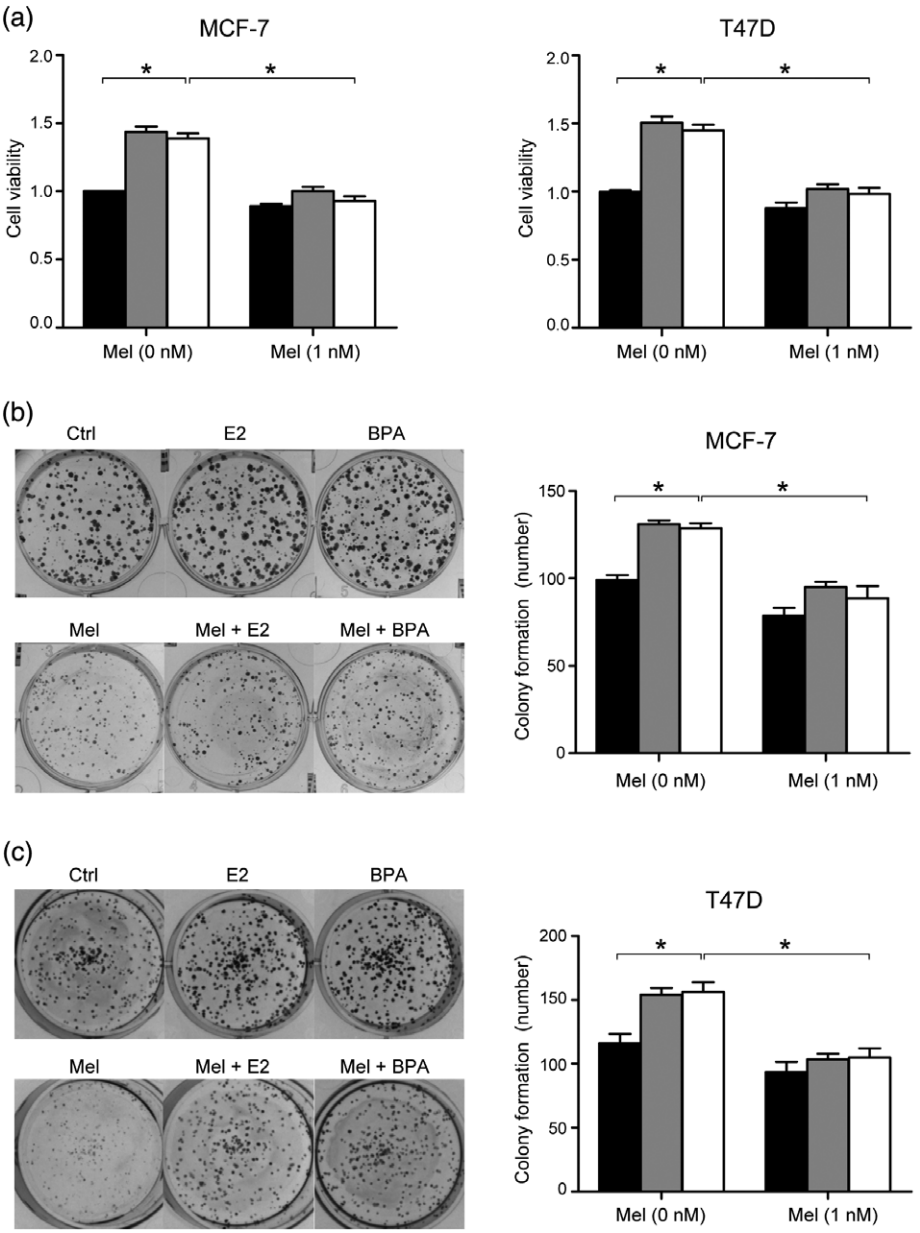

Figure 1 Melatonin could block the survival and proliferation of estrogen receptor-positive breast cancer cells induced by bisphenol A (BPA). (**a**) MTT assay was performed to evaluate cell proliferation. Cells were incubated for 96 hours and the value of optical density 490 nm was measured as described in the MTT method. The bars represent the average value and the standard deviation of at least three independent experiments; $*P < 0.05$. The survival and proliferation of MCF- 7 (**b**) and T47D (**c**) cells were detected by colony formation assay as described above, the proliferative ability was enhanced by BPA (100 nM) and E2 (10 nM), while this enhancement could be blocked by melatonin (1 nM). The bar graph showed statistical analysis of colony numbers for at least three experiments; $*P < 0.05$. Ctrl, E2, BPA.

Melatonin decreases the activity of ERE stimulated by BPA

The change of ER coactivators indicates the alteration of ERE activity.[19] Thus, we considered whether ERE activity was also involved in the modulation of melatonin on BPA-induced proliferation. To verify this hypothesis, we performed luciferase reporter gene assay in MCF-7 and T47D cells (Fig 4). The cells were transiently transfected with pERE-E1B-luc plasmid. Luciferase reporter activity demonstrated that melatonin could effectively inhibit ERE activity promoted by BPA.

Discussion

BPA is a food contact material that is used as composition of plastics for the manufacture of food packaging, beverage bottles, kitchenware, wall of cans, and so on. Exposure to BPA could be constant in daily life, and severely threatens female and male health.[1,6,10] *In vitro* and *in vivo* investigations have revealed the connection between BPA with BC, which was found to be caused by the estrogen-like properties of BPA including interacting with ER and activating ERE.[20–22] Notably, BPA is competent to raise the levels of ERα and progesterone receptor, recruiting ERα to the

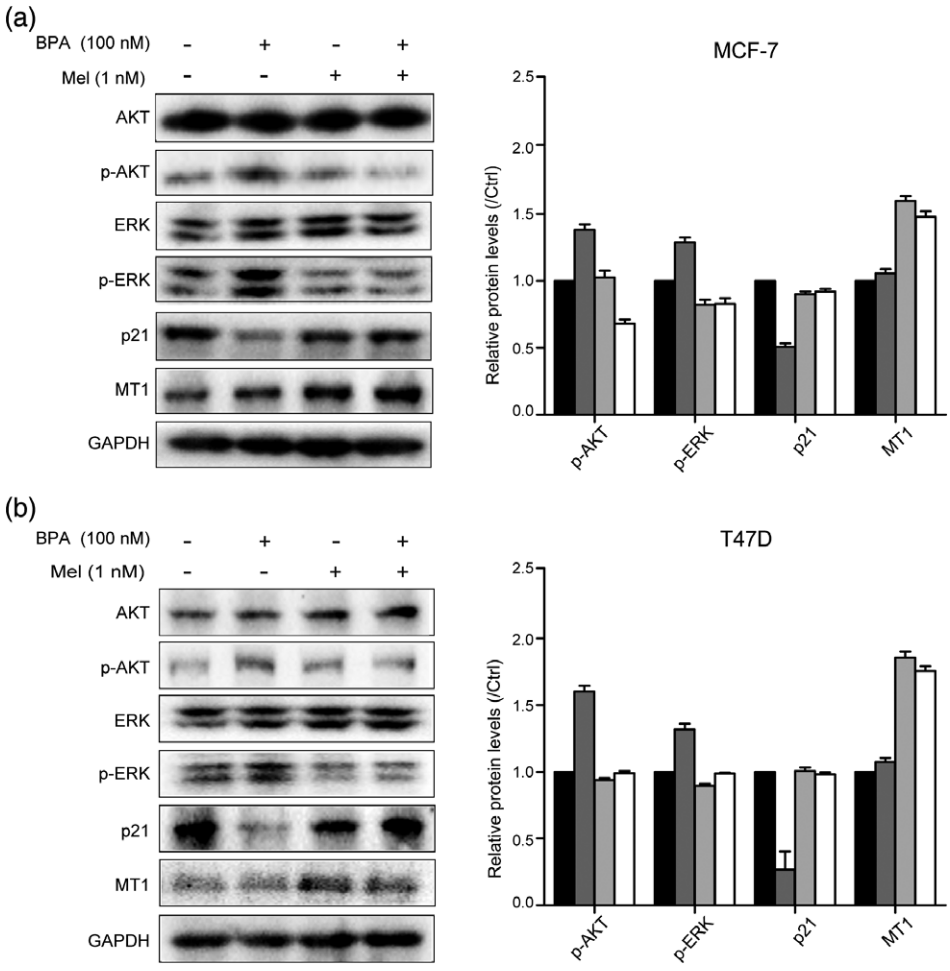

Figure 2 Melatonin is able to modulate the levels of estrogen receptor-related key proteins under treatment of bisphenol A (BPA). (**a**) MCF-7 and (**b**) T47D cells were starved for 24 hours, and then treated with BPA at a dose of 100 nM or melatonin at a dose of 1 nM for 48 hours. The protein levels of p-AKT, p-ERK, p21, and MT1 were detected by western blotting assay. The histogram showed relative levels changed under treatment. The bars represent the average value and the standard deviation of at least three independent experiments. Ctrl, BPA, Mel, BPA+Mel.

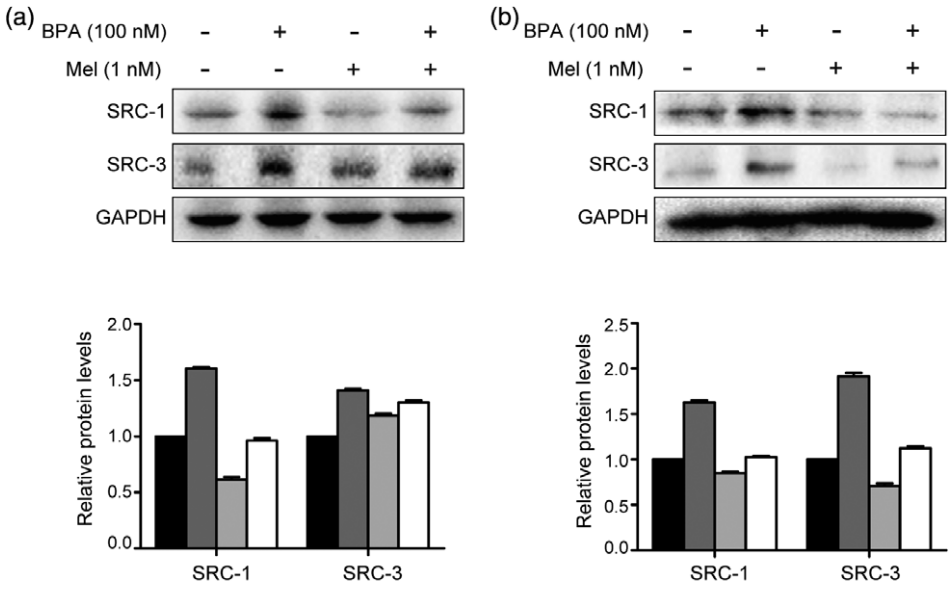

Figure 3 Melatonin is capable of inhibiting bisphenol A (BPA)-elevated steroid receptor coactivator (SRC)-1 and SRC-3. (**a**) MCF-7 and (**b**) T47D cells were treated with BPA (100 nM) and melatonin (1 nM) for 24 hours after starved by Dulbecco's modified Eagle's medium without phenol red and fetal bovine serum. Western blotting assay was performed to detect a change of proteins. The histogram shows relative levels changed under treatment. The bars represent the average value and the standard deviation of at least three independent experiments. Ctrl, BPA, Mel, BPA+Mel.

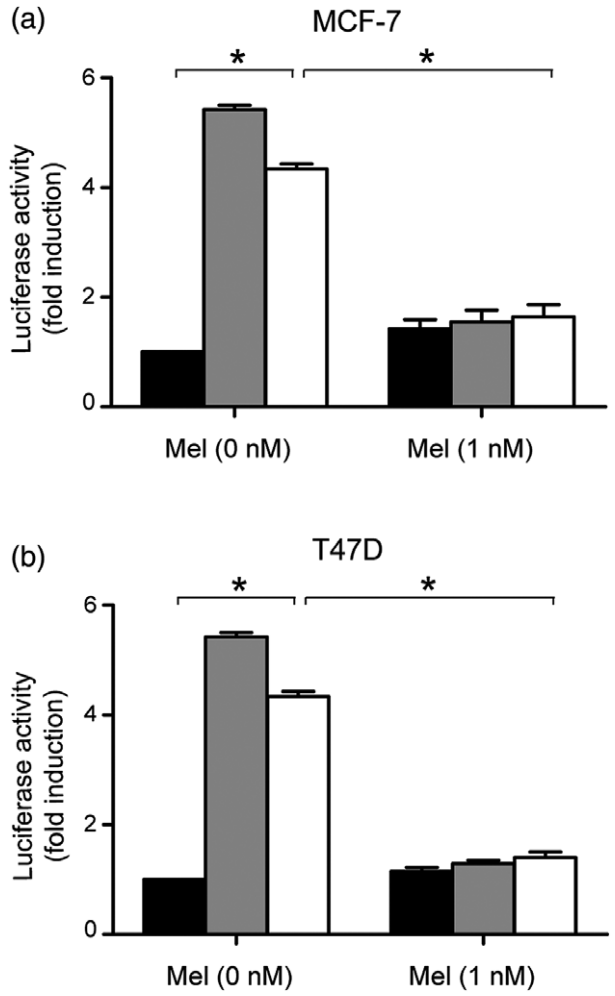

Figure 4 Melatonin decreased the activity of estrogen response element stimulated by bisphenol A. (**a**) MCF-7 and (**b**) T47D cells were transfected with 1 μg of pERE-E1b-luc reporter plasmid (estrogen response element-driven reporter plasmid). After 12 hours' starvation, cells were treated with 10 nM E2, 100 nM BPA, and 1 nM melatonin. The luciferase activity was measured as indicated and the data were showed as fold change compared with untreated cells (control group), which was arbitrarily indicated as 1. The bars represent the average value and the standard deviation of at least three independent experiments; $*P < 0.05$. Ctrl, E2, BPA.

promoter of estrogen-responsive genes in a dose-dependent pattern, which was similar to the effect attained by E2 treatment.[23] It is reported that oral exposure to BPA could induce mammary carcinoma in rats through the activation of the AKT pathway.[24] However, whether there exists a potential agent that suppresses BPA-induced cell proliferation in BC remains unknown.

As an endogenous hormone, melatonin not only works as a regulatory factor for circadian rhythm, but is also involved in angiogenesis and tumor growth.[25–27] Amongst the characteristics of melatonin, we focus on its function as an anti-neoplastic substance on hormone-associated tumors. Our data demonstrated that melatonin was capable of suppressing BPA-induced proliferation in ER^+ BC cells. We have known that BPA could mimic estrogen to form a complex with ER and then activate ERE, as well as the MAPK and PI3K/AKT signaling pathways.[28–32] Here, we found that melatonin could abolish BPA-elevated phosphorylation of ERK and AKT. Numerous studies have shown the crucial role of melatonin receptor MT1 in melatonin-induced anticancer events.[33–36] In the present study, we observed an obvious elevation of MT1 after treatment of melatonin, which may be involved in the abolishment of BPA-associated cell proliferation.

Given the estrogen-like properties of BPA, we are concerned as to whether BPA promoted BC cell proliferation through ER coactivators, SRCs. Strikingly, we for the first time found that BPA was capable of elevating the expression of SRC-1 and SRC-3. Furthermore, the activity of ERE could be efficiently increased by BPA treatment. However, melatonin could significantly disrupt the BPA-elevated SRCs expression and ERE activity, which could be regarded as the mechanisms of melatonin in the suppression of BPA-induced BC cell proliferation.

In the present study, we find that melatonin could reverse BPA-induced proliferation of BC cells via reducing the phosphorylation of ERK and AKT, as well as upregulating the level of cell cycle progression blocker, p21. Most importantly, melatonin blocks the activation of ERE triggered by BPA, possibly through downregulating ER coactivator, SRC-1 and SRC-3. Thus, we propose that melatonin could be used as a promising medication for BPA-associated BC progression.

Acknowledgments

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Disclosure

No authors report any conflict of interest.

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