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Estrogen receptor-dependent effects of bisphenol a

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Abstract

Bisphenol A (BPA), commonly used as building block of polycarbonate plastics, significantly affects human and animal health interfering with the action of natural hormones. Within BPA disrupting effects, a mitogenic activity and, consequently, an increased incidence of neoplastic transformations has been reported in exposed organisms. Among the several mechanisms proposed for the mitogenic BPA effects, its ability to bind to estrogen receptors (ERα and ERβ) deserves particular attention. Aim of this work is to investigate ERα- and ERβ-dependent mechanisms underlying BPA proliferative effect. Binding assay confirms that BPA binds to both ERs. Cell vitality assay and Western blot analysis of protein involved in cell proliferation demonstrate that BPA acts as a double side disruptor of estrogenic effects. In fact in the presence of ERα, BPA mimics E2, increasing cell proliferation. On the contrary, in the presence of ERβ, BPA acts as an E2 antagonist preventing the hormone-induced cancer cells apoptosis. These two divergent aspects could act synergistically in the exposed organisms leading to the disruption of the balance between proliferation and apoptosis typical of E2 effects.

Introduction

Several pesticides, phtalates, and several phenolic compounds, such as alkylphenols and biphenyls including biphenyl methane, also known as bisphenol A (2,2-bis(4-hydroxyphenyl) propane, BPA, are listed in the group of endocrine disruptors (EDs). EDs represent heterogeneous and widely distributed hormone-like chemicals [1, 2], able to interfere with the synthesis and the action mechanisms of natural hormones, consequently affecting organism homeostasis, at concentrations below their toxicity threshold (i.e., in the μM range). BPA, a small monomer (228 Da), is commonly used as building block of polycarbonate plastics for baby and water bottles, epoxy resins coating food containers, and white dental sealants [3]. BPA serves also as an additive in other types of plastics, such as polyvinyl chloride (PVC), medical tubing, toys, water pipes, and polyethylene terephthalate [4]. The ester bond between the BPA molecules is unstable, it is disrupted by heat and by acidic or basic conditions [5, 6]; therefore BPA leaches into food or beverages in contact with the plastics at a high rate. Animal, including humans, exposure can arise from several sources [3, 7, 8]. Several reports describe adverse effects of BPA at doses lower than the current level considered safe by the U.S. Environmental Protection Agency (EPA) [4, 9-17]. Of particular relevance, epidemiological studies have highlighted the correlation between the increase of BPA level in the environment and the incidence of cancer (i.e., breast cancer and prostate cancer) in humans [4, 18-23]. Several mechanisms have been proposed for the mitogenic BPA effects. Among others, estrogen receptors α and β (ERα and ERβ), ligand-activated transcription factors belonging from nuclear receptor super-family [23], have been reported as the foremost molecular mediators of the *in vitro* and *in vivo* effects exerted by BPA [4, 24-27]. BPA binding to ERs [28], modulates ER transcriptional activity [24-27,] inducing elevated proliferation and chromosomal alterations [29-33]. However, ERα and ERβ are also localized at the plasma membrane where they initiate 17β-estradiol (E2)-induced rapid signals crucial for the E2-induced modulation of proliferation in several cancer target cells [9, 23, 34]. Therefore, it is reasonable to suggest that BPA binds to ERα and ERβ producing changes in these ER-activated rapid signals. Few data address this ability of BPA [9, 35, 36]. Aim of this work is to investigate ERα- and ERβ-dependent mechanisms underlying the proliferative effect of BPA.

Materials and methods

Radiometric binding assays by using [^{3}H]-E2 as tracer was performed as previously reported [35]. Different human cancer cell lines were used as experimental models. Particularly, we used HeLa cells (cervix adenocarcinoma), devoid of any ER isoform, and DLD-1 cells (colon carcinoma), endogenously expressing ERβ. In order to render HeLa cells responsive to E2 or BPA, Hela cells were transiently transfected with the human pSG5-hERα vector of expression [37]. An empty vector, pCMV5, was used as control [37]. Furthermore, to evaluate the transcriptional activity of ERβ, DLD-1 cells were transfected with the reporter plasmid containing the promoter of complement component 3 gene (pC3), retaining a natural estrogen responsive element (ERE) [35]. Six hours after Hela or DLD-1 transfection with lipofectamine,

the medium was changed and after 24 h cells were stimulated as indicated. To evaluate BPA effect on transfected HeLa and DLD-1 cells, the cells were plated in 96-well culture plates and stimulated with either vehicle (DMSO:PBS, 1:1) or different concentrations of E2 (10^{-11}M-10^{-6}M) or different BPA concentrations (10^{-8}M-10^{-3} M), or E2 (10^{-8}M) in the presence of different BPA concentrations (10^{-6}M-10^{-3}M). After 24h or 30h, respectively, HeLa and DLD-1 cell vitality was assessed by using the XTT reaction solution according to the manufacturer's instructions. To evaluate ERβ transcriptional activity, DLD-1 cells were stimulated with different BPA concentrations (10^{-6}M-10^{-3}M) or with 10^{-5}M BPA in the presence of 10^{-8}M E2 for 6h [35]. Finally the activation state of kinases involved in ER-dependent signal transduction pathway was determined by Western blot as previously described [35, 36, 38].

Results

BPA binds either human recombinant ERα and ERβ proteins [35, 36, 39] with a value of the dissociation equilibrium constant (*i.e.*, Kd) for BPA binding to ERβ about three fold lower than that for BPA association to ERα [35, 39] (Tab. 1). Since Kd value for BPA binding to ERα and ERβ is approximately three-fold higher than that for E2 association [35, 36, 39], the concentrations of E2 and BPA used in these studies were 10^{-8}M and 10^{-5}M, respectively (Tab. 1).

	ERα Concentration (M)	ERβ Concentration (M)
E2	$(2.1\pm0.5)\times10^{-10}$	$(3.5\pm0.5)\times10^{-10}$
BPA	$(1.17\pm0.3)\times10^{-6}$	$(4.8\pm0.6)\times10^{-7}$

Table 1. Kd value for E2 and BPA binding to ERα and ERβ.

As already reported [37, 38], E2 stimulation increases ERα-transfected HeLa cell number and reduces DLD-1 cell number (Tab. 2). BPA (1-10μM) mimics E2 effect, only in ERα-transfected HeLa cells [35] (Tab. 2) and this effect is mediated by ERα since no effect has been observed in empty vector-transfected HeLa cells or in ERα-transfected HeLa cell pretreated with ER inhibitor ICI (Tab. 2). On the contrary, in DLD-1 cells, BPA alone has no effect at any of the tested concentration, whereas DLD-1 cell costimulation with E2 and BPA results in a loss of E2-induced reduction in cell vitality (Tab. 2). Also in this case, no effect has been observed when DLD-1 cells are pretreated with ER inhibitor ICI, confirming the ERβ involvement (Tab. 2).

	HeLa Cells (empty)	HeLa Cells (ERβ) % of variation	DLD-1 Cells (endogenous ERβ)
E2	0	+150*	-50*
BPA	0	+133.3*	0°
E2+BPA	-	-	0°

Table 2. E2 and BPA effect on empty- or transfected Hela cells and DLD-1 cells. Cells have been treated 30 h with 10 nM E2 or 10 μM BPA. Data, mean of six different experiments, are reported as% of variation with respect to the control. SD is less than 10%. P> 0.001 was calculated with Student's t test with respect to vehicle (*) or 17β-estradiol (°, E2).

	P-AKT[a] (Empty)	P-ERK[a] (Empty)	P-AKT[a] (ERα)	P-ERK[a] (ERα)	P-AKT[b]	P-ERK[b]	P-p38[b]
E2	0	0	+400*	250*	0	0	+300*
BPA	0	0	+400*	200*	0	0	0
E2+BPA	nd	nd	nd	nd	0	0	0

Table 3. E2 and BPA effect on ERK and AKT activation and protein level in empty- or transfected Hela cells and DLD-1 cells. Hela cells (a) and DLD-1 cells (b) have been treated 60 min with either vehicle (control) or 10 nM E2 or 10 μM BPA. Data, mean of four different experiments, are reported as% of variation with respect to the control. SD is less than 10%. P> 0.001 was calculated with Student's t test with respect to vehicle (*).

Successively we correlate the (in)direct BPA-induced proliferative response to the ERβ- and ERα-mediated extranuclear signal activation. BPA induces the rapid (60 min) ERK and AKT phosphorylation only in ERα-transfected HeLa cells (tab.3). These effects are not present in empty vector-transfected cells and are completely prevented by ICI, confirming that BPA-dependent AKT and ERK activation requires ERα presence (Tab.3).

Furthermore, the inhibition of these kinases, by using specific ERK or AKT inhibitors, impairs BPA-induced proliferation in ERα-transfected HeLa cells, confirming the involvement of these pathways in the BPA-induced ERα-mediated cell proliferation (Tab.4). In DLD-1 cancer cells, instead, BPA prevents the E2-induced increase of p38 phosphorylation (Tab. 3). According to the loss of p38 activation, BPA alone does not affect caspase-3 activation and PARP cleavage but prevents E2 effects on pro-apoptotic protein activation when cells are co-stimulated with BPA and E2 [36]. These data are strongly supported by our previous finding demonstrating that BPA impairs ERβ association with the signaling protein p38, decoupling ERβ from the downstream signals important for the E2-induced pro-apoptotic cascade [36]. Interestingly, BPA also blocks ERβ transcriptional activity. In fact, while the E2 treatment induces a two-fold increase of the pC3 promoter activity (tab.4), BPA alone has no effect on the transcription of a gene containing the ERE element (pC3), but, when added with E2, BPA prevents the E2-induced ERβ transcriptional activity (Tab. 4).

Previously, we reported that both genomic and extranuclear ERβ activities are required for the E2-induced increased expression of ERβ, which represents an important step for the E2-mediated protection against colon cancer cell growth [40]. In fact, ERβ decreased level is associated with colonic tumorigenesis and loss of malignant colon cell differentiation [40]. Accordingly to the BPA ability to block both genomic and extranuclear activities of ERβ (Tab. 4), we observe the loss of E2-induced increased expression of ERβ (Tab. 4).

	pC3 promoter activity	ERβ level
	% of variation	
E2	+114.3*	+300*
BPA	0°	0°
E2+BPA	0°	0°

Table 4. E2 and BPA effect pC3 promoter activity and ERβ protein level in DLD-1 cells. Cells have been treated 60 min or 24h with either vehicle (control) or 10 nM E2 or 10 μM BPA or E2 plus BPA. Data, mean of six different experiments, are reported as% of variation with respect to the control. SD is less than 10%. P> 0.001 was calculated with Student's t test with respect to vehicle (*) or 17β-estradiol (°, E2).

Discussion

Here we report a new ERα- and ERβ-mediated mechanism underlying the proliferative action of BPA in cancer cells. In fact, BPA proliferative effect in cancer cells requires ERα, acting as a mimetic of E2 in the presence of this ER isoform by the activation of rapid and non-genomic pathways important to drive cells to proliferation. On the other hand BPA acts as an E2 antagonist in the presence of ERβ, blocking the E2 ability to reduce cancer cell proliferation. As a whole, these two mechanisms allow us to depict BPA as a double sided E2 disruptor, which promotes tumor incidence in breast and other target organs that predominantly express ERα but inhibits the E2 protective effects in the ERβ-expressing colon.
These two divergent aspects could act synergistically, thus increasing the E2-disrupting potential of this widespread environmental polluter. Finally, the BPA-induced modulation of ER activities points to the rapid E-induced mechanisms as the most susceptible targets of endocrine disruptors.

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