Low Doses of Bisphenol A Promote Human Seminoma Cell Proliferation by Activating PKA and PKG via a Membrane G-Protein–Coupled Estrogen Receptor

Adil Bouskine,1,* Marielle Nebout,1,* Françoise Brucker-Davis,1,2 Mohamed Benahmed,1 and Patrick Fénichel1,2

1Institut National de la recherche Médicale (INSERM) U895, Team 5—Environment and Reproduction: Genomic and Nongenomic Mechanisms, University of Nice-Sophia-Antipolis, Faculty of Medicine, Nice, France; 2Department of Reproductive Endocrinology, University Hospital of Nice, Nice, France

Endocrine-disrupting chemicals (EDCs) are hormone-like agents present in the environment that may alter the endocrine system of wildlife and humans. In particular, xenoestrogens have been hypothesized to be involved in developmental, reproductive, and malignant diseases by mimicking the natural hormone 17β-estradiol (E2) and interfering with endogenous endocrine regulation at specific periods, such as during fetal growth. Several organochloride pesticides—polychlorinated biphenyls (PCBs), phthalates, and bisphenol A (BPA)—used in the chemical industry have been considered as estrogenic EDCs. However, all of these EDCs have a very weak affinity for binding through the classical nuclear estrogen receptors (ERs), 1,000-2,000 times lower than that of E2 (Bonefeld-Jørgensen et al. 2001; Craik et al. 1998; Massaad and Barouki 1999). Studies of the nuclear transcriptional regulatory activities of nonphysiologic estrogens have mostly been unable to explain the actions of these chemicals in mediating endocrine disruption in animals and humans at the low picomolar or nanomolar concentrations widespread in the environment (Calafat et al. 2005; Vandenberg et al. 2007). In the last few years, EDCs have been reported to act through hormone-independent mechanisms (Welschons et al. 2006) or through a nongenomic activation of membrane-initiated signaling pathways via membrane forms of ERs (Alonso-Magdalena et al. 2005; Bulayeva et al. 2002). Indeed, there is now convincing evidence that estrogens, in addition to the classical regulation of estrogen-responsive genes via nuclear ERs, are able to trigger rapid membrane activation of a variety of second-messenger–mediated signal transduction pathways (Kelly and Levin 2001; Vasudevan and Pfaff 2007), with possible implications for cell proliferation, apoptosis, or survival (Levin 2002). However, the nature of these membrane ER(s), their relation to the classical ERs, and the precise signaling pathways that are activated remain to be elucidated (Manavathi and Kumar 2006; Vasudevan and Pfaff 2007). Moreover, fetal exposure to xenoestrogens is believed to be involved in male reproductive and developmental pathogenesis. Diethylstilbestrol (DES), a potent synthetic estrogen used as an abortifacient drug in the 1970s, has a well-known deleterious effect in adults exposed in utero (Newbold et al. 2006). DES can produce different developmental or carcinogenic effects in rodents (Newbold 2004) when given during specific developmental windows, including fetal or perinatal periods, such as cryptorchidism or breast, prostate, or endometrial cancers. However, although indirect epidemiologic data show a constant increase in testicular cancer in young men (Huystje et al. 2007) and an increased relative risk via professional exposure to persistent organic pollutants, no experimental model has validated the possible carcinogenic role of exposure to xenoestrogens in developing testicular germ cell cancer (Rajpert-De Meyts 2006).

BPA, initially produced like DES as a synthetic estrogen (Dodds and Lawson 1936), has been rapidly and widely used as a cross-linking chemical in the manufacture of polycarbonate plastic and epoxy resins. Because of incomplete polymerization and degradation of the polymers by exposure to higher than usual temperatures, BPA leaches out from food and beverage containers (Biles et al. 1997; Krishnan et al. 1993; Le et al. 2008), as well as from dental sealants. BPA is found in the serum, milk, saliva, and urine of humans at nanomolar concentrations (Calafat et al. 2005; Olea et al. 1996; Sun et al. 2004; Vandenberg et al. 2007). Remarkably, BPA has been measured in amniotic fluid at concentrations 5-fold higher than those measured in maternal plasma (Ikezuki et al. 2002). Fetal and perinatal exposures to BPA in rodents have been shown to affect the brain, mammary gland, and reproductive tract, including hormone-dependent cancer (Durando et al. 2007; Ho et al. 2006; Maffini et al. 2006; Markey et al. 2001; Munoz-de-Toro et al. 2005). Although BPA induces an estrogenic effect through classical nuclear ERs at high

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Address correspondence to P. Fénichel, University Hospital of Nice, 06202 Cedex 3, France. Telephone: 33-04-92-03-55-19. Fax: 33-04-92-03-54-25. E-mail: fenichel.p@chu-nice.fr

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concentrations and with a reduced affinity relative to E2 (Gaido et al. 1997; Krishnan et al. 1993; Perez et al. 1998), it also is able to trigger a nongenomic effect in prostate tissues, endothelial, and hypophyseal cells and in breast cancer cells by initiating rapid responses at low concentrations (Alonso-Madgalena et al. 2005; Bulayeva and Watson 2004; Nadal et al. 2000; Noguchi et al. 2002).

We recently reported that E2 coupled to bovine serum albumin (E2-BSA) stimulated the proliferation of human seminoma cells (JKT-1) in vitro through a G-protein–coupled nonclassical membrane ER (GPRC) (Bouskine et al. 2008). In the present study, we investigated the hypothesis that BPA could stimulate seminoma cell proliferation through such a nongenomic action. We observed a promoting effect of BPA on seminoma cells through a rapid activation of cAMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase (PKG) signaling pathways via a GPCR, illustrating that xenestrogens, suspected to act as deleterious factors in breast and prostate cancers, could also act in this nongenomic pathway as possible promoting agents in testicular germ cell cancer.

Materials and Methods

Cell culture and cell proliferation assay.

JKT-1, a human testicular pure seminoma cell line developed from the testis of a 40-year-old man (Kinugawa et al. 1998), expresses the classical gonadal steroid receptor ERα and E2 (Roger et al. 2004), and expresses the classical gonadal steroid receptor ERα and E2 (Roger et al. 2004). E2, the major steroid secreted by the ovary, differentiates the seminoma marker (Roger et al. 2004) and expresses the classical gonadal steroid receptor ERα and E2 (Roger et al. 2004). In the present study, we investigated the hypothesis that BPA could stimulate seminoma cell proliferation through such a nongenomic action. We observed a promoting effect of BPA on seminoma cells through a rapid activation of cAMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase (PKG) signaling pathways via a GPCR, illustrating that xenestrogens, suspected to act as deleterious factors in breast and prostate cancers, could also act in this nongenomic pathway as possible promoting agents in testicular germ cell cancer.

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tested (10^{-9} \text{ M}). However, E_2-BSA plus BPA induced the same effect of each alone, indicating a lack of synergic or antagonistic effect and likely a similarity in the activated pathways (Figure 2).

**BPA activates PKA and CREB in JKT-1 cells.** Activation of PKA was necessary for BPA to promote JKT-1 cell proliferation because H89, a specific PKA inhibitor, totally prevented an increase in cell proliferation (Figure 3A). Extracellular stimuli elicit changes in gene expression in target cells by activating intracellular protein kinase cascades that phosphorylate transcription factors within the nucleus. CREB is one of these factors that activates gene transcription after the phosphorylation of serine 133 induced by a variety of protein kinases, including PKA and extracellular-signal-regulated kinase 1/2 (ERK1/2) (Shaywitz and Greenberg 1999). Using an anti–phospho-CREB antibody that recognizes phosphorylated serine 133, we observed a very rapid (5 min), BPA-induced activation of CREB in JKT-1 cells, with maximum activation at 30 min (Figure 3B). This activation was PKA dependent because H89 completely abolished CREB phosphorylation (data not shown).

The cell cycle regulator Rb is phosphorylated during BPA-induced promotion of JKT-1 cells. Rb is a nuclear factor that participates in the regulation of the cell cycle, interfering with cyclin action when nonphosphorylated. This suppressive effect is prevented through phosphorylation of Rb. In JKT-1 cells, BPA induced a rapid (4 hr) and intensive phosphorylation of Rb (Figure 4), leading to Rb inactivation during BPA stimulation.

ICI does not prevent BPA-induced JKT-1 proliferation. We recently reported that JKT-1 cells express ER-β but not ER-α (Rogé et al. 2005). By immunofluorescence, subcellular fractionation, and Western blot, we found that the ER-β receptor had an apparent intracytoplasmic localization without any evident membrane location (Bouskine et al. 2008).

To determine whether ER-β was involved in the estrogenic or xenoestrogenic activation of JKT-1 cell proliferation, we tested the effect of ICI, a pure ER antagonist. ICI completely counteracted the suppressive effects of E_2 and DES on JKT-1 cell antagonism (Figure 5), supporting an ER-β–dependent mechanism. However, ICI did not prevent the promoting effect of BPA or of E_2-BSA, supporting our hypothesis that the rapid effect induced by these two ligands was likely not dependent on a classical ER (Figure 5).

**BPA stimulates proliferation in JKT-1 cells through a G-protein–coupled receptor (GPCR).** GPCRs have been proposed to be involved in triggering membrane action of steroids (Thomas et al. 2006), including estrogen (Filardo et al. 2002; Kelly and Wagner 1999). PKA activation is usually a G_{G_{a}} protein-dependent mechanism, so we tested the effect of NF449, a G_{G_{a}} inhibitor (Figure 6). NF449 blocked the promoting effect of BPA, illustrating the G_{G_{a}} dependence of the PKA activation and the G-protein–coupled nature of the receptor involved in BPA stimulation. We have previously shown that E_2-BSA, which triggers an effect quite similar to that of BPA, also induces a G_{G_{a}}-dependent activation of the mitogen-activated protein kinase (MAPK)/ERK1/2 pathway (Bouskine et al. 2008). For this reason, we also studied the effect of PTX, an inhibitor of G_{G_{a}}/G_{G_{b}} protein, during BPA-induced JKT-1 proliferation. This
toxin prevented the BPA-induced increase of cell proliferation (Figure 7).

**PKG pathway but not MAPK pathway is activated by BPA in JKT-1 cells.** BPA promotion of JKT-1 cells did not seem to involve ERK1/2 activation because PD, an inhibitor of MAPK kinase, did not prevent BPA-enhanced proliferation (Figure 8). We therefore tested the PKG pathway, which is known to be Gαq/Gαs dependent and is involved in BPA activation of calcium influx in pancreatic islet α cells (Alonso-Magdalena et al. 2005). KT5823, an inhibitor of PKG activation, prevented BPA-induced JKT-1 proliferation (Figure 6).

These results strongly support the participation of a membrane GPCR involving both the Gαq and Gαs subunits. Figure 9 summarizes the signaling pathways activated during BPA-induced JKT-1 proliferation.

**Discussion**

In this article we demonstrate for the first time that very low doses of BPA (picomolar or nanomolar) stimulate human seminoma cell proliferation by allowing a rapid, nongenomic, membrane-initiated activation of PKA and PKG signaling pathways associated with phosphorylation of the transcription factor CREB and the cell cycle regulator Rb. This promoting effect, similar to the one observed with E2-BSA but not with E2 alone (Bouskine et al. 2008), was triggered independently of classical ERs through a membrane receptor belonging to the GPCR family. The low concentrations of BPA able to produce such an effect give this observation environmental relevance and support the hypothesis of a possible contribution of xenoestrogenic fetal exposure to testicular germ cell carcinogenesis.

Estrogens classically mediate their action after binding to nuclear receptors that act as transcription factors to modulate the activity of target genes by interacting with several DNA response elements. In addition to their ability to mediate gene transcription, estrogens also elicit rapid nontranscriptional effects by membrane-mediated signaling pathways leading to calcium influx (Chaban et al. 2004), cAMP (Abraham et al. 2003) or nitric oxide production, phospholipase C activation, or inositol phosphate generation (Le Melay et al. 1997). The MAPK/ERK1/2 pathway can also be rapidly activated by estrogens in various cell types, such as endothelial (Chen et al. 2004), adipocytes (Dos Santos et al. 2002), neuroblastoma (Watters et al. 1997), or breast cancer cell lines (Migliaccio et al. 1996). Membrane activation of these rapid signaling cascades will then modulate gene transcription (Vasudevan and Pfaff 2007). We have recently reported that human seminoma cells express both classical ER-β (Roger et al. 2005) and a membrane nonclassical estrogen GPCR (Bouskine et al. 2008). E2 has a high affinity for ER-β and triggers a suppressive effect in JKT-1 cells, whereas E2 coupled to BSA, which prevents membrane crossing, binds to an ncmER and promotes cell proliferation by activating rapid cell signaling, including PKA and MAPK pathways (Bouskine et al. 2008). BPA has a low affinity for ER-β, as described in several models, with a 1,000-fold weaker affinity than E2, and activates pancreatic islet, hypophyseal, or endometrial cells through an ncmER (Alonso-Magdalena et al. 2005; Bulayeva and Watson 2004; Nadal et al. 2000; Noguchi et al. 2002). In JKT-1 cells, the differential affinity toward both receptors may explain the dose–response curve observed. At high micromolar concentrations, BPA may trigger a suppressive effect via ER-β as does E2, which neutralizes the non genomic effect. At low concentration (10^{-9} M), this genomic effect is absent, allowing the nongenomic effect to be displayed because of the high affinity of BPA for the ncmER. When mixed together at this low concentration, BPA and E2 are mutually antagonistic, whereas DES, also a potent ligand for nuclear ER, at this low concentration only moderately counteracts the nongenomic BPA effect, possibly for conformational reason. Our model illustrates the paradoxical inverse U-shaped curve, explaining effects at very low doses (Brucker-Davis et al. 2001), that has been described for BPA in several models (Maffini et al. 2006; von Saal and Hughes 2005; Welshons et al. 2006), which could be produced by two different ERs and two different, genomic and nongenomic, mechanisms.

We propose that the promoting effect occurs through nongenomic transduced activation of the PKA/CREB and the PKG pathways, as illustrated by the very rapid phosphorylation of CREB and the inhibition of both CREB phosphorylation and proliferation obtained with the PKA antagonist H89 and the PKG inhibitor NF449. Phosphorylated CREB will regulate cell-cycle-controlling genes as demonstrated by Rb phosphorylation. Estrogenic activation of the PKA/CREB pathway through an ncmER has already been described in several models (Belcher et al. 2005; Filardo et al. 2002; Quesada et al. 2002). Concerning ERK activation, Wozniak et al. (2005) assessed the rapid changes in intracellular calcium levels induced by xenoestrogens in a pituitary tumor cell line and found that multiple membrane-initiated signaling pathways were activated. The differential patterns presented seemed to depend on the structure of the xenoestrogens and the conformation obtained with the membrane ER (Wozniak et al. 2005). In particular, BPA was one of the EDCs tested that did not
induce ERK activation (Wozniak et al. 2005), as in our seminoma cell model. In another model of pancreatic islet α cells, Alonso-Magdalena et al. (2005) showed that estrogens induce a rapid calcium influx through several pathways, including PKG. We therefore tested the PKG pathway, which involves for its activation a Gαq subunit, and found its contribution in BPA-induced JKT-1 proliferation as well, likely through a rapid intracellular calcium increase. Then, we showed that the BPA proliferation-promoting effect on seminoma cells is in fact needed the two subunits Gαq and Gβγ, which acted not in an opposite but in a complementary fashion, as already described in other models (Daaka et al. 1997) and supported in JKT-1 cells by the use of specific inhibitors. Indeed, activation of both the PKA and PKG pathways seemed to be necessary for the BPA-induced promoting effect, as we have previously shown for E2-BSA with the two PKA and ERK pathways (Bouskine et al. 2008). BPA, when mixed with E2-BSA, showed the same proliferative effect as each compound alone, without any synergistic or antagonistic effect. Despite the mild difference in the activated protein kinases, it is likely that this promoting effect induced by E2-BSA and BPA is mediated via the same GPCR membrane receptor, an ncmER.

Increasing evidence from different tissues and cell types has suggested that there are multiple mechanisms through which estradiol can stimulate rapid intracellular signaling (Kelly and Levin 2001; Manavathi and Kumar 2006). However, one of the main questions remains the nature of the coupled receptors. Different studies of nonclassical estrogen signaling in a variety of target cells, such as endothelial, neuronal, and pituitary cells (Kim et al. 1999; Li et al. 2003), have strongly suggested that nuclear classical ER or ER-like proteins are candidates (Pedram et al. 2006) for the membrane ERs. In our model, however, this membrane receptor is unlikely to be a classical ER because ICI failed to inhibit cell proliferation; JKT-1 cell membranes do not express ERβ as we previously reported (Bouskine et al. 2008); and E2 and DES, a potent synthetic estrogen that binds to ER, do not trigger a promoting but a suppressive effect. Filardo et al. (2002) showed that estrogen-induced ERK activation may occur in human breast cancer cells that do not express either ER-α or ER-β. GPR30, an orphan GPCR, has been proposed as a nonclassical estrogen receptor able to stimulate cancer cells (Albanito et al. 2007; Filardo 2002; Revankar et al. 2005; Thomas et al. 2005; Vivasqua et al. 2006). Thus, GPR30 is a candidate for our ncmER in JKT-1 seminoma cells, activated by E2-BSA or BPA and able to transduce the PKA and ERK or PKG signaling pathways. Moreover, GPR30 has been identified recently in mouse spermatogonia and has also been involved in estrogenic germ cell proliferation control (Sirianni et al. 2008). Seminoma cells are considered to be issued from transformed gonocytes or undifferentiated spermatogonia (Rajpert-De Meyts 2006). Thus, we propose that GPR30 could represent the ncmER in JKT-1 seminoma cells, able to activate PKA, ERK, or PKG pathways. Studies of its expression, precise localization, and involvement in triggering an estrogenic promoting effect in human seminoma cells and human fetal or adult germ cells are now under way in our laboratory.

It is now possible, as we recently proposed (Bouskine et al. 2008), to more comprehensively describe actions of estrogens at low concentrations on human seminoma cells, involving two different and opposite effects: first, a predominant long-lasting, suppressive effect, antagonized by ICI, thus likely mediated by ER-β, which involves a classical nuclear genomic pathway controlling cell cycle gene expression (Roger et al. 2005); and second, a rapid, nongenomic promoting effect triggered by PKA and ERK activation, which involves not a classical ER (not antagonized by ICI or tamoxifen), but rather a PTX-responsive nonclassical membrane estrogen GPCR (Bouskine et al. 2008). The resulting impact on germ cells may depend on the relative expression of both receptors (ER-β and GPCR), the endogenous concentration of E2, and the respective binding affinity of the estrogenic compounds. In the presence of both receptors, as in JKT-1 seminoma cells, the suppressive effect of E2 may remain predominant because of a rapid dephosphorylation of ERK1/2 by an ER-β-dependent expression of protein phosphatase 2A, as described in neonatal rat cerebellar neurons (Belcher et al. 2005). In contrast, in JKT-1 cells, BPA does not need the MAPK pathway, but rather the PKG pathway, which would have a more potent effect on the nongenomic pathway because BPA has a higher affinity for this receptor. Furthermore, human gonocytes, which do not express the active ER-β1 isof orm until the prenatal period (Gaskell et al. 2005), may be exclusively sensitive to the membrane-mediated promoting effect if they do not express the ncmER as do mouse spermatogonia (Sirianni et al. 2008). Progressive expression of ER-β1 during the perinatal period will prevent proliferation and enhance differentiation into spermatogonia. However, excessive fetal exposure to xenoestrogens with high affinity for the nonclassical estrogen GPCR, as shown here for BPA, may promote abnormal proliferation of gonocytes through the nongenomic pathway and thus contribute to malignant germ cell transformation, leading, as proposed by Skakkebaek et al. (1998), to carcinoma in situ and then to testicular germ cell cancer, the most frequent cancer of the young men with an increasing incidence.

This GPCR-mediated nongenomic action therefore represents a new basis for evaluating xenoestrogens such as BPA that could interfere with the developmental programming of fetal germ cell proliferation and/or differentiation when it crosses the placenta. Testing of EDCs will require a cell model that expresses this receptor alone and/or together with nuclear ERs.

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