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Chapter 5

BPA SIGNALING THROUGH NON-CANONICAL RECEPTORS

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ABSTRACT

Bisphenol A (BPA; 2,2-bis(4-hydroxyphenyl)propane) is one of most prevalent and studied endocrine disruptors. In rodents, fishes and amphibians, BPA has been shown to produce reproductive defects sometimes associated with sexual differentiation or with a possible feminization of males. Given that, and as BPA was originally synthesized to be an estrogenomimetic, it is not surprising that estrogen receptors (ER) have been tested as possible mediators of BPA effects. However recent data suggest that BPA may act via ER-independent mechanisms. For example, recently, it has been shown that BPA elicit an effect on mouse fetal Leydig cells in the absence of ERα. In addition, BPA was proven to decrease testosterone production in the human developing testis even though diethylstilbestrol, a potent ER-agonist, had no effect at this stage. Thus additional signaling pathways are likely targets of BPA. While the affinity of BPA for ER is weak (~1000 fold lower in comparison to estradiol), BPA has been proposed as a ligand for other receptors such as the estrogen-related receptor gamma (ERRγ) or the G protein-coupled estrogen receptor (GPER). In particular, the orphan nuclear receptor ERRγ has been shown to bind BPA at the nanomolar range with high specificity. Recently, it has been shown that BPA positively regulates the transcriptional activity of human ERRγ. Recent data have shown that these *in vitro* effects were significant in terms of *in vivo* activities. These data suggest that ERRγ is indeed an *in vivo* target of BPA and that this receptor may be implicated in BPA-induced developmental defects. Other endocrine receptors such as TR or AR are also possible targets of BPA although the *in vivo* relevance of these alternative receptors is still debated. Therefore these data extend the

range of pathways perturbed by this compound and reveal that its potential harmful effects are larger than expected. This prompts for a re-evaluation of the risk assessment of BPA.

INTRODUCTION

Chemicals termed endocrine disrupting compounds (EDC) are believed to mediate their effects through disturbing the normal endocrine systems. Based on the long notorious records about EDC-induced alteration of the reproductive functions, most EDCs were challenged for pro-estrogenic or anti-androgenic properties via Estrogen Receptors (ER) and the Androgen Receptor (AR) [1-5]. And indeed when one seeks for such an effect it is often observed in many cases at very high concentrations. However evidence for the involvement of additional or alternate pathways are emerging from transgenic models, genomic analysis etc.... The existence of such alternative pathways may account for the effects observed at much lower concentrations as well as for the non-monotonic dose-response often observed with EDCs [6-9].

Understanding the signalling that, when disturbed by a given EDC induce health defect is a challenging goal; yet, it is an obligatory step for assessing the risk linked to EDCs exposure. EDCs are retrieved as complex mixtures and at low doses in our daily environment. Thus, the identification of the signalling pathway(s) targeted by a given EDC should allow the evaluation of the effect of additional substances on the same pathway and the consideration for potential additional and even synergistic effects. It should also allow a faster screen of potentially harmful compounds through the use of high or medium throughput systems. However, this requires the use of *in vivo* models in which toxicity is indeed observed and the demonstration that: i) the proposed signalling is present and active in the altered tissues or cell, ii) this signalling is regulated by the EDC in the target tissue and iii) EDC toxicity is lost when such a signalling is rendered inactive. Unfortunately, for most of these compounds the primary targets involved in their *in vivo* toxicity remain elusive [10, 11].

In this line, the case of Bisphenol A (BPA) is compelling. BPA has long been considered as an estrogeno-mimetic. This is mostly due to many reports involving BPA in defects of the male reproductive function and to some feminizing effects in vertebrates [12-14]. As an example, it was reported that BPA prevents some defects associated to the loss of aromatase, the enzyme responsible for estrogen synthesis [15]. On the contrary, several recent studies reported some BPA-effect mediated by alternate signalling, sometimes clearly independent of canonical estrogen signalling. In addition, biochemical and structural data points towards BPA being a ligand of very weak affinity for estrogen receptors (ER), poorly compatible with environmentally relevant doses. Some of the proposed alternate signalling pathways have been shown to be activated by very low doses in the range of the nanomolar. In this chapter we review the data that link *in vitro* and *in vivo* the effects of BPA to its various receptors: first, ERs but also the two main alternative targets GPR30 and ERRγ. We also present rapidly the other nuclear receptors discussed as possible targets. Lastly, we discuss the difficulties associated with the identification of the *in vivo* receptors mainly due to the links between these pathways and we broaden this discussion in the context of other EDC molecules.

CANONICAL ESTROGEN RECEPTORS (ER)

As mentioned above, many studies regarding BPA have focused on the reproductive system. An abundant literature shows that BPA displays estrogenic activity in a number of experimental systems and has the potential to adversely affect reproductive function and development in both human and wildlife (see [11, 12, 14, 16-19] and references therein). Therefore it is not surprising that estrogen receptors have been considered very early on as possible mediators of BPA effects. Estrogen receptors (ER, also termed NR3A or ESR) have initially been considered has a family of two receptors ERα and ERβ binding specifically estrogens and acting as transcription factors binding to consensus estrogen response element (ERE) (review in [20]). More recently fast non-genomic estrogenic signalling has been described via cytosolic or membrane-associated ER [21].

It has been shown that BPA binds and activates the mammalian ERα and ERβ although with an affinity 10 000 weaker than for their natural ligand 17β-estradiol [22-24]. The affinity constant of BPA for ERα and ERβ has been estimated at 0.2 and 0.04μM respectively [22, 25]. This has been confirmed in teleost fishes such as zebrafish which contains one ERα and two ERβ genes, ERβ-A and ERβ-B resulting from a genome duplication [26, 27]. In this species BPA have been shown to behave as a partial agonist in the micromolar range for ERα and ERβ-B, but not for ERβ-A [28]. In accordance with these observations, a number of estrogenic effects of BPA, among which the classic vitellogenin induction, have been demonstrated in several teleost fish models [14, 29, 30]. All these *in vitro* data may therefore suggest an effect of BPA via the estrogen receptors, even if the affinity observed *in vitro* is barely compatible with the relatively low doses at which BPA exert some effects *in vivo* [31].

However some data indicate that the situation may be more complex than anticipated. For example several transcriptomic analyses of BPA effects have suggested that the gene regulatory signature elicited by this compound is readily different from the one of 17β-estradiol, the natural ligand of ERs, although this depends of the organism and the tissue studied [32-38]. Additionally, transcriptomic analysis of developing mammary gland in WT and ERα KO mice suggested that ERα may not play a major role as a mediator of the BPA effects in this tissue [39]. Of course these differences may be explained at least in part by the different biodisponibility, stability or metabolism of the two molecules but this is unlikely to explain all the difference observed.

Far more important, the reproductive effects of bisphenol A in the complete absence of estrogen receptors have never been described in mouse or in fishes. Therefore we have no direct proof that indeed the effects of BPA are ER-dependent even if there is indeed some indications that goes in that direction. However approaches based on the usage of pharmalogical agent suggest the involvement of ER. In several cells or tissues, a well know ER antagonist, ICI-182780, was proposed to counteract BPA effects [40-44]. As an example, in the adult rat testis BPA was proposed to alter meiotic progression and decrease sperm production [43]. These effects are similar to those observed with oestradiol and BPA-toxicity is prevented by the co-administration of ICI, therefore suggesting that ERs may be critical for the BPA effect.

Strikingly, in either mouse or zebrafish, the two most commonly used functional models to study BPA action, genetic evidences of ER involvement in BPA effect remain scarce. Three studies using transgenic mice, observed no effect of BPA in ER KO mice. First, BPA

(1 nM) was proposed to promote arrhythmia specifically in the heart of female mice [45]. Such an effect was absent in the myocyte of ERβ KO mice. Second, alteration of early oogenis in response to BPA exposure has been reported in mouse, rat, rhesus monkey and human [46-49]. *In utero* exposure to BPA disturbs meiotic progression in mouse early oocytes. In this model, BPA had no effect in ERβ KO mice ovaries albeit unexposed ERβ KO oocyte already displayed similar meiotic abnormalities [46]. This example therefore suggests that ERβ is required to elicit BPA effect. Lastly, in pancreatic β-cells, BPA was reported to increase insulin content and this effect was lost in cells from ERα$^{-/-}$ mice [40]. Interestingly, in this study the authors proposed that BPA action in β-cells involved ERKs/MAP kinase activation, thus possibly through non-genomic signalling.

In contrast, there are several indications of *in vivo* effects of BPA for which the presence and/or activity of ERs appears dispensable, providing evidence that the effects are mediated by another receptor. A recent investigation of BPA effect on steroidogenesis in the fetal testis indicated that BPA decreases the production of testosterone at this stage [13]. This effect has been reported in three species: mouse, rat and human using an organ culture model. In this system, 10 nM of BPA lowered the production of testosterone of the human fetal testis. Interestingly, diethylstilbestrol (DES), a potent estrogenomimetic, had no effect on the testosterone production of the human fetal testis in the same model, possibly due to the absence of ERα in human fetal Leydig cells [50]. In rodents, ERα is described in Leydig cells and DES effectively decreases the production of testosterone in the developing testis [51]. In the mouse, ERα was evidenced as physiologically involved in the regulation of the testosterone production of the testis. Indeed, ERαKO, but not ERβKO, have an elevated production of testosterone during fetal life [52]. Of interest, in ERαKO mouse fetal testis, BPA still inhibited the production of testosterone with a similar efficiency when compared to wild-type, indicating that ERα is dispensable for the observed effect [13]. Altogether in this model the BPA effect on steroidogeneis is likely independent of the ERs, albeit the identification of the signalling involved is currently unknown. Similarly, in breast cancer cells BPA in the nanomolar range was proposed to confer chemoresistance, even in the absence of ERα or in the presence of the ER antagonist, ICI-182780 [53]. Such effects possibly independent of ER were also described in human endometrial stromal fibroblasts, ovarian antral follicles and hippocampal neurons *in vitro* [54-56]. In overall, it is really astonishing that despite numerous endpoints are described altered in response to BPA very few, apart those above-mentioned, were investigated in ER KO. Even more striking, even if the phenotype of the ERα/ERβ double KO mice is described and these mice are available for study, to our knowledge no study has ever been published describing the effect of BPA in the absence of both ERs. The same situation holds for zebrafish even if in this species the existence of 3 ERs further complicate the genetic analysis.

Taken together these various cases suggest that both ER-dependent and ER-independent actions of BPA exist. In this line, it will be of interest do define whether the ER-dependent action are due to a direct effect of BPA binding on ER or to indirect effect such as possibly an altered steroid production. In frame with the second hypothesis it can be reported that BPA has been proposed to modulate the expression and the activity of the estrogen synthesizing enzyme, P450 aromatase/CYP19, in several cell types and tissues [57-61].

It is worth reporting here that an emerging literature is reporting long-term effects following BPA exposure during fetal and/or post-natal life associated with DNA

hypomethylation in mice and rats [62-64]. DNA methylation is one of the most common epigenetic marks, epigenetics being considered as any inheritable change that does not change the DNA sequence. As an example, Hoxa10, a homeobox gene involved in uterine organogenesis, has its expression increased and its methylation decreased following *in utero* exposure of female mice to BPA [64]. Of interest the authors identified that this decrease of DNA methylation led to an increase in ERE-driven gene expression and propose this as new mechanism for BPA action that may thus permanently increase estrogen sensitivity.

MEMBRANE BOUND ESTROGEN RECEPTORS

G protein-coupled receptor 30 (GPR30, now termed GPER, G protein-coupled estrogen receptor 1) is a seven transmembrane-domain receptor that has been evidenced to bind to estradiol and BPA with an IC50 respectively of 17.8 nM and 630 nM [65]. It could thus mediate some of the fast non-genomic response to estrogen. GPR30 couples to G proteins and modulates second messenger pathways, notably cAMP and calcium. Most of the studies that investigated GPR30 role relied on cancer cell lines and on the use of pharmacological agents. One of the main studies that proposed some BPA effects mediated through GPR30 was performed using the JKT-1 cell line [66]. JKT-1 is a cell line derived from a testicular germ cell tumor initially proposed as a seminoma but now rather classified as an embryonic carcinoma [67]. In this cell line very low doses of BPA (1 pM to 1 nM) increased proliferation. Interestingly, oestradiol conjugated to BSA was also able to stimulate JKT-1 proliferation by triggering a rapid and membrane-mediated activation of ERK1/2 and protein kinase A (PKA) [67]. Importantly, this effect could not be reversed by ICI nor reproduced by DES (that is inactive on GPR30) but BPA-induced cell proliferation was prevented in presence of a PKA inhibitor. The authors also demonstrated that BPA effect on cell proliferation was similar to the one produced by a specific agonist of GPR30 (G1) and reversed by a GPR30 antagonist G15 [68]. Additional studies proposed a similar role for GPR30 in human and mouse cell lines such as breast cancer cells [69] and Sertoli cells [70]. In the first study [69] the authors reported that BPA induced the proliferation and migration of SKBR3 breast cancer cells that lacks ERs but expresses GPR30 and that this effect was cancelled when GPR30 expression was silenced by shRNA. In the second study [70] exposure of Sertoli TM4 cells to G15 abolished the BPA-induced proliferation of the cells. Of note, BPA action at low doses (1 nM) appears poorly compatible with its proposed IC50 for this receptor (630 nM) and this point will warrant future investigations.

ERK/MAPK involvement is proposed to be downstream of GPR30 (as for extra-nuclear ER signalling). In this line, it is interesting to note that in numerous tissues and cell lines it is reported that exposure to BPA induces an activation of ERK/MAPK pathways. Additionally, inhibitors of MAPK were proven to prevent some BPA effects. This was evidenced *in vitro* in spermatogonial cell line, GC1 [71], in neurons [72, 73], in SKBR3 breast cancer cells [69] and in Sertoli cells and cell line TM4 [70, 74]. As an example in SKBR3 cells, 0.1 μM of BPA triggered a rapid (30 min) phosphorylation of ERKs, similarly to that induced by G1 and abolished in presence of shRNA directed against GPR30, suggesting that GPR30 is required for ERK activation.

Lastly it is worth noticing that in some cases, though GPR30 and MAPK appeared associated to BPA action, it is also reported that ICI counteracted the effects of BPA [70, 75]. This complicates the situation and may suggest that a cross-talk exists between ER and GPR30. Such a cross talk has indeed been suggested [76, 77] albeit this one requires further clarification. Interestingly, it is proposed that BPA could activate ER by phosphorylating ERα through GPR30 and MAPK/ERK pathways [75]. Such a possible cross talk could explain how both GPR30 and ER might mediate BPA effect. However these demonstrations rely in large part on the use of agonists and antagonists, and thus one should remain cautious about their interpretation. As an example, ICI has been reported to bind also GPR30, even though acting as an agonist [78].

Of note, though GPR30 is widely expressed, the Gpr30KO mice were proven viable and fertile [79, 80]. To our knowledge, no study has yet investigated BPA toxicity in those mutants. However, G1 agonist and Gpr30KO phenotype display some opposing effects reminiscent of those of BPA previously described depending of ER using ERKO mice (see above). G1 stimulates insulin release [81] while in Gpr30KO pancreatic islet insulin is decreased [82].

G1 increased heart rate [76] and Gpr30KO female displayed a cardiovascular phenotype [82], albeit this last one might have numerous origins. Though GPR30 is widely expressed in reproductive tissues, its role has not been investigated in the fetal ovary. However *in vitro* knock-down of GPR30 impaired follicle formation stimulated by estradiol [83] and *in vivo* exposure to BPA has also been proposed to alter follicle formation [48, 84]. This suggests that although ER was proven required for some BPA action this should not exclude GPR30 involvement, until demonstrated.

ESTROGEN-RELATED RECEPTOR γ (ESRRG/NR3B3)

The estrogen-related receptors (ERR α,- β and-γ; NR3B 1-3) form a group of orphan nuclear receptors closely related to ERs [85, 86]. There are three *ERR* genes in human that are critical regulators of metabolism but that are also important in the control of cell proliferation, hence an implication in cancer. Of interest, ERRs share target genes, co-regulator proteins and sites of action with the ERs [87, 88].

Even more relevant, if ERRs are still orphan receptors in the sense that their endogenous ligands, if any, are unknown, their activities can be regulated by pharmacological ligands, some of which being also ligands for ERs. Indeed, if natural estrogens do not interact with ERRs, the synthetic compounds 4-hydroxytamoxifen (4-OHT) and DES are ligands of ERRβ and γ that behave as inverse agonists [89-91]. Given their potential importance as drug targets it is not surprising that a large effort has been devoted to the search of agonist or antagonist ligands of ERRs and this explain the relatively large number of molecules that were identified (see Table 1 and below). Among this diversity BPA is one of the more interesting and, very significantly, one that display a high affinity and selectivity.

Table 1. Known molecules interacting with ERRs.

Note that the binding is most often not demonstrated. Given the variety of the studies the concentration indicated are merely indicative and were not determined using the same assays

Ligand	ERR	Concentration	Comment	Reference
XTC790	α	IC_{50}: 400 nM	Inverse agonist. Coactivator release. ERRα selectivity demonstrated	Busch et al., 2004, Willy et al., 2004
Compound 1a	α	Kd: 770 nM	Inverse agonist. Coactivator release. Crystal structure. Effect on β and γ not tested.	Kallen et al., 2007
Toxaphene	α	ca. 10 μM	Inverse agonist.. Effect on β and γ not tested.	Yang and Chen, 1999
Chlordane	α	ca. 10 μM	Inverse agonist. Effect on β and γ not tested.	Yang and Chen, 1999
Thiazolidinedione "29"	α	IC_{50}<150 nM	Inverse agonist. Coactivator release. Crystal structure. Inactive on γ. Effect on β not tested.	Patch et al., 2011
Troglitazone	α and γ	ca. 10 μM	Inverse agonist. Effect on β not tested.	Wang et al., 2010
Kaempferol	α and γ	ca. 2 μM	Inverse agonist. Effect on β not tested.	Wang et al., 2009, Huang et al., 2010
Genistein	α	ca. 10 μM	Activation. Some effect on β ? Inactive on γ. Not observed in some studies	Suetsugi et al., 2003, Abad et al., 2008, Huang et al., 2010
Daidzein	α>β>γ	ca. 10 μM	Activation. Not observed in some studies	Suetsugi et al., 2003, Abad et al., 2008, Huang et al., 2010
Biochanin A	α >β, γ	ca. 10 μM	Activation. Not confirmed	Suetsugi et al., 2003,
6,3',4'-trihydroxyflavone	α >β, γ	ca. 10 μM	Activation. Not confirmed	Suetsugi et al., 2003
DES	β, γ >α	EC_{50}: 700 nM (γ and β)	Inverse agonist. Coactivator release. Inactive on α. Crystal structure for γ.	Tremblay et al., 2001a, Coward et al., 2001, Greschik et al., 2004

Table 1. (Continued)

Ligand	ERR	Concentration	Comment	Reference
GSK4716 (Hydrazone)	β, γ	EC_{50}: 1 μM	Agonist. Inactive on α. Crystal structure	Zuercher et al., 2005, Wang et al., 2006
GSK9089 (Hydrazone)	β, γ	EC_{50} ca. 10 μM	Agonist. Inactive on α.	Zuercher et al., 2005
DY131 (Hydrazone)	γ>β	ca. 1-10 μM	Agonist. Inactive on α.	Yu and Forman, 2005
4-hydroxytamoxifen (OHT)	γ>β	EC_{50}: 50 nM (γ) and 150 nM (β) Kd for γ: 35 nM	Antagonist. Inactive on α. Crystal structure for γ.	Coward et al., 2001, Tremblay et al., 2001b, Greschik et al., 2004, Wang et al., 2006
Tamoxifen	γ>β	EC_{50}: 400 nM (γ) and 950 nM (β)	Antagonist. Inactive on α.	Coward et al., 2001
GSK5182 (Tamoxifen annalogue)	γ	79 nM	Antagonist. Crystal structure. Effect on α and β not tested.	Chao et al., 2006
Tetra-aryl-substituted alkenes	γ	> 10 μM	Antagonist. Effect on α and β not tested.	Koh and Park, 2011
BPA	γ	IC_{50}: 131 nM, Kd: 5.5 nM	Agonist. Reverse the antagonist action of OHT Crystal structure.	Takayanagi et al., 2006, Matsushima et al., 2007, Abad et al., 2008
BPA and Phenol derivatives	γ	μM range	Same than BPA	Okada et al., 2007, Li et al., 2010
4-chloro-3-methyl phenol	γ	ca. 700 nM	Compete for OHT binding but no change in activity described. Crystal structure.	Abad et al., 2008
Cholic acid	γ	N.D.	Fortuitous ligand identified during crystalization. Change in activity unknown. Effect on α and β not tested.	Greschik et al., 2004
Apigenin	γ	ca. 10 μM	Inverse agonist. Binding.	Huang et al., 2010
Luteolin	γ	ca. 10 μM	Inverse agonist	Huang et al., 2010

References [89-113].

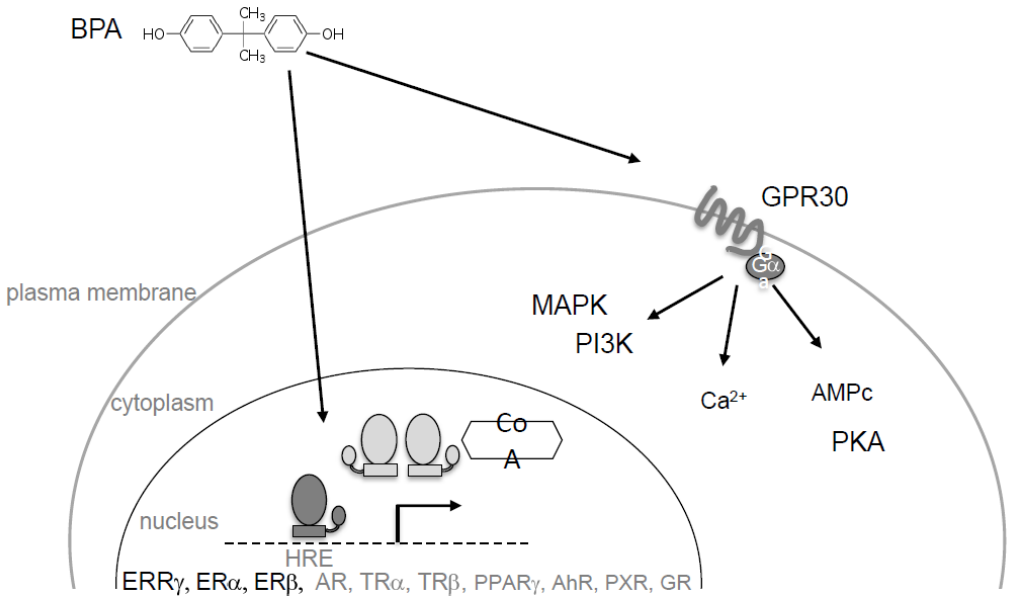

Figure 1. Bisphenol A is proposed to signal through nuclear receptors and/or membrane bound receptors. GPR30 (GPER) is the main receptor suspected to mediate BPA action through extra-nuclear compartment. On the other hand, many nuclear receptors signaling through possibly close Hormone Response Elements(HRE) and/or sharing co-activator (coA) are evoked to mediate BPA action.

It is the group of Tasuyuki Shimohigashi in Japan that have suggested for the first time a relationship between BPA and ERRs and that have accumulated most of the data on this aspect. First, *in vitro* BPA was shown to strongly bind to ERRγ by showing that it can displace radiolabelled OHT from the ligand-binding domain of the receptor [92]. Interestingly, in the same study BPA was shown to be unable to regulate directly the activity of ERRγ which displays a high constitutive activity but was able to reverse the antagonist action of OHT. In presence of increasing concentration of BPA the inhibitory activity of OHT is relieved. This defines a new class of ligands for NR that have no activity per se but is able to counteract the effect of an antagonist. BPA binds to ERRγ, with a Kd of 5.5 nM [111], a much more relevant dose than the micromolar expected needed to bind to ERα or ERβ [114]. This may therefore provide a clear substrate to explain the low-dose effects of BPA. The binding was fully demonstrated by the observation that specific mutations disrupt the interaction and, even more convincingly by the determination of the 3D structure of the ERRγ ligand binding domain complexed to BPA [112, 113, 115, 116]. This interaction between BPA and ERRγ has also been observed on non mammalian ERRs. In zebrafish there are two ERRγs, called ERRγ-A and γ-B and both of them are affected by BPA in a very similar manner than mammalian ERRγs [117]. In addition a slight effect on ERRβ was observed whereas there is no effect on ERRα and ERRδ, a fish specific ERR [117]. In even more distant animals, namely the insect *Chironomus riparius* and the mollusk *Marisa cornuarietis* BPA was shown to activate ERR even if the effect is modest and seen only at high concentration [118, 119].

The relevance of this BPA-ERRγ interaction was further substantiated by exploration of the specificity of the interaction. Indeed Okada et al., 2008 tested several BPA derivatives for

their ability to interact with human ERRγ. These authors found that only one of the two phenol hydroxyl groups of BPA was essential for full binding. Of note in compounds in which one of the two methyl groups were removed (such as BPE) a similar binding was observed (IC50 of 8.14 nM when compared to 9.78 nM for BPA [111]).

Taken together these data show very convincingly the *in vitro* relevance of the BPA/ERRγ interaction. Clearly BPA is a high affinity specific ligand for ERRγ. But how does this translate to the *in vivo* situation? Is there any evidence that BPA action *in vivo* could be mediated by ERRγ?

The first indication came from a study using the human trophoblastic cell line, JEG-3 in which it was shown that BPA treatment at 0.1 μM impaired cell proliferation and induce apoptosis [120]. Interestingly the same authors showed that a siRNA directed against ERRγ partially abolished this effect. This suggest that the antiproliferative effect of BPA on this cell line is, at least in part mediated by ERRγ, although the disruption of other receptors, such as ERs have not been studied. A recent analysis by Shimonogashi group suggested that effectively ERRγ is expressed and active in human placenta, a tissue in which BPA accumulates [121]. In another interesting study 10 nM BPA was shown to increase spine density in adult hippocampal rat neurons [56]. This effect was not abrogated by ICI treatment but blocked by OHT. Since OHT is an antagonist of both ERs and ERRs whereas ICI block only ERs this suggest that this effect is ERRγ-dependant, an observation that is in accordance with the expression of this gene in hippocampal neurons.

The second evidence came from zebrafish. In this species, a new phenotype elicited by BPA was recently described: During development BPA induces a specific malformation of the otic vesicle, namely the formation of abnormal aggregates of otolithes, the small mineralize structure that is critical for equilibrium [122]. This effect was originally demonstrated to be ER-independent since it is not mimicked, nor inhibited, by treatment with ER specific agonists or antagonists. Recently, it was shown that this specific effect of BPA was ERRγ-dependent [117]. Two lines of evidence converge to this conclusion: first the same BPA derivatives than those tested *in vitro* by Okada et al., 2008 [111], were tested *in vivo*. It was shown that these different compounds can induce a similar otolith phenotype. Strikingly, the binding affinity of these derivatives to the zebrafish ERRγ correlates with their ability to induce otolith malformations suggesting that to elicit this phenotype a compound must bind to ERRγ. The second piece of evidence is a more direct genetic evidence: if the two zebrafish ERRγs are depleted using morpholino injection a loss of the otolith is observed suggesting that ERRs effectively control otolith formation in zebrafish. If lower dose of morpholinos are injected in zebrafish embryos to only partially deplete the ERRγs, the BPA dose response curve is shifted to the right: that is higher doses of BPA are required to observe the effect. In contrast the overexpression of ERRγ allows to mimic the BPA phenotype, an observation that is not surprising given that ERRγ exhibit a strong constitutive transcriptional activity. Interestingly the co-treatment with low dose of BPA that are inactive in wild-type embryo, induce an otolith phenotype in the injected embryos. Taken together these data suggest that in zebrafish embryos ERRγs are necessary and sufficient to allow the effect of BPA on the otic vesicle [117]. It is interesting to note that a recent analysis have shown that ERRγ plays a role in the maintenance of hearing in human and mouse, suggesting that indeed, in several vertebrate ERRγ is important in the otic system [123]. This reinforces the relevance of the observed BPA-induced phenotype in zebrafish.

Of course it would be of prime importance in this context to study the effect of BPA in the context of an ERRγ null mice. However, ERRγ is expressed in metabolically active and highly vascularized tissues such as heart, kidney, brain, and skeletal muscles and is a key regulator of multiple genes linked to both fatty acid oxidation and mitochondrial biogenesis in cardiac muscles [124] (reviewed in [125]). As it has a number of important physiological function, the ERRγ-/- mice die during the first week of life [126]. It will therefore not be easy to study the effect of BPA in the context of those mice and the use of conditional mutant will be required. This would be however very important to perform since there are more and more evidences showing that in addition to its reproductive effect BPA has also a number of metabolic effects, for example on glucose metabolism [127] and given the important metabolic function of ERRγ it could be envisaged that some of these effects are mediated by this receptor rather than by ERs. On the other hand the involvement of ERRγ in numerous BPA-induced defects observed in zebrafish should also be informative about the extent of this additional mechanism of action.

Of interest ERRγ is a key transcriptional regulator of mitochondrial metabolism and biogenesis and numerous alteration following BPA exposure point toward an increase in mitochondrial dysfunction and oxidative stress. As an example in TM4 Sertoli cell line high doses (10^{-5} M) of BPA elevated oxidative stress while low doses (10^{-8} M) BPA promoted energy metabolism by elevating mitochondrial activity and intracellular ATP [128]. Oxidative stress is caused by reactive oxygen species (ROS) due to decreased activities of scavenger proteins or by dysfunction of the mitochondrial respiratory chain pathway. ROS can damage various cell components such as unsaturated lipids, proteins, and nucleic acids. Growing evidence points toward BPA-deleterious effects being associated with oxidative stress. Indeed, in mouse tissues and in various cell lines such as Neuro2a and GC1 cells, BPA induces ROS production, modulates antioxidant enzymes or compromises mitochondrial function [129-131]. The molecular mechanism of ROS production by BPA remains unclear and one may suspect that a deregulation of ERRγ could be involved even if this is not demonstrated yet.

OTHER ENDOCRINE-RELATED TARGETS OF BPA

Additional receptors such as androgen receptor (AR), glucocorticoid receptor (GR), peroxisome proliferator-activated receptor (PPAR), aryl hydro-carbon receptor (AhR) and thyroid receptor (TR) were proposed to mediate BPA activities though no evidence remain weak (for review [132]). Studies of amphibian metamorphosis reported that BPA disrupt T3-signaling in tadpoles [133] and *in vitro* studies have shown that BPA can also antagonize T3 activation of the TR [134, 135]. Of note, halogenated derivatives of BPA such as tetra-bromo-bisphenol A (TBBPA) used as flame retardant are likely more potent regulators of TR and have also been proven to alter thyroid hormone signaling in the Xenopus laevis Embryo [136]. BPA has also been reported to disrupt PPAR signalling [137] and similarly TBBPA appears as a convincing regulator of this pathway too. Indeed those activate PPARγ in zebrafish and in reporter cell lines [138].

In silico study proposed that BPA could bind to human GR [139] and *in vitro* exposure of the 3T3-L1 cell line indicated that BPA promotes adipogenesis through favouring the GR

activation [140]. Several *in vitro* studies reported that BPA has anti-androgenic activities inhibiting AR activation, interaction and transcriptional activity [141-144]. On the other hand, other authors observed no anti-androgenic properties of BPA [145] in similar *in vitro* models. Lastly, luciferase reporter gene assays also indicate that BPA affected the AhR activation [143]. Additionally in AhRKO antral follicle the inhibitory effect of BPA (110 μM) on growth was proven attenuated, albeit BPA at higher doses was still efficient [146]. On the other hand this might simply reflect the remarkable increase of AhR that has been observed in other tissues following *in utero* exposure to BPA [147].

Lastly, it is interesting to note that BPA have been shown to be an agonist for human PXR at 2 μM, but not for mouse PXR. The authors have identified the key residues within PXR ligand binding domain responsible for this difference [148]. They further illustrate the *in vivo* relevance of this regulation, especially for cardiovascular effects of BPA using PXR humanized mouse model [149]. Given that PXR control the expression of CYP detoxifying enzyme in the liver it would also be interesting to know if such a regulation may indeed contribute to a increased clearance of BPA in the body but this is still unknown to our knowledge.

Altogether those data propose that numerous nuclear receptor related to multiple endocrine pathways might be altered following BPA exposure. However a careful examination of the relevancy of these information in *in vivo* model is required.

CONCLUSION

From all these observations three conclusions emerge: i) some effects of BPA are effectively mediated by the ERs, though it is not obvious that those are direct targets; ii) there are two well identified alternative targets: GPR30 and ERRγ that must be taken into account; iii) other possible receptors from the nuclear receptor superfamily may also participate to the BPA response. Because the action of these various receptors are often linked due to multiple cross-talks it will be difficult to decipher in detail the contribution of each of them. We are clearly facing a very difficult issue.

ERs Are Not the Only Targets of BPA

We have presented the relatively few clear cases in which BPA action was firmly shown to be mediated by ERs. Because the EDC field was convinced of the link between EDC action and reproduction and since ERs are important players in regulating reproduction, this lack of data relating BPA action and ER have often not been seriously considered. However, given the amount of work devoted to BPA action it is quite striking and this must induce to question the relevance of ERs as BPA targets. Indeed if *in vitro* the binding of BPA to estrogen receptors is clearly demonstrated, the affinity when compared to its endogenous ligand (17β-estradiol) is low (ca. 10 000 fold weaker). This means only high dose (over the micromolar range) of BPA could effectively act via ER. Therefore one of the burning questions around BPA effect, the one of the low-doses effects (below the acceptable daily intake dose of 50 mg/kg/day), should be discussed in the light of this conclusion.

The existence of low-dose effect was often not accepted because of the lack of a possible mechanism of action [17]. The fact that some effects of BPA are demonstrated to be ER-independent clearly suggests that low dose effects may be mediated by alternative receptors. Therefore these ER-independent effects should now be taken into account for risk assessment and policy making by regulatory agencies. This also raises the problem of using of pre-defined positive controls. The systematic use of DES or other estrogenic compounds seems questionable and thus their absence in a study appears no longer as a reason for not taking such studies into account.

Table 2. Calculations of receptor occupancy versus hormone concentration for two receptor affinities: A receptor with a low Kd (e.g. ERRγ; 5.5nM) and a receptor with a high Kd (e.g. ERs; 55nM)

The Kds of ERα and ERβ for BPA are 200 and 40 nM respectively that is before and after the high Kd value we selected for this simulation. This simulation was done following the Table 1 from Welshons et al., 2003 [9].

BPA Concentration	Receptor occupancy	
	Kd 5.5 nM (ERR)	Kd 55nM (ERs)
5500 nM (=5.5μM)	99.9%	99%
550nM	99%	91%
55nM	91%	50%
5.5nM	50%	9%
0.55nM	9%	1%
0.055nM (=55pM)	1%	0.1%

The existence of several potential receptors for a unique molecule such as BPA should also be considered to explain the non-monotonic dose-response curve often observed with BPA (see [132] and references therein). Indeed if two different receptors, one with high affinity and one with a lower one both act antagonistically on the same biological output (let's say cell proliferation in a given organ) one can observed U-shaped curves when the concentration of compound is increased. We believe that this may allow explaining many of those examples described in the literature and that the existence of several receptors for BPA should be taken into account in those situations.

ERRγ and GPR30 Are Likely Alternative BPA Receptors

As discussed above there is now convincing *in vitro* and *in vivo* evidence to allow to firmly conclude that GPR30 and ERRγ play an important role in mediating some BPA actions. The Kd of BPA for human ERRγ is 5.5nM whereas the Kd for ERα and ERβ has been estimated at 200 and 40nM respectively [22, 25, 114]. As illustrated in Table 2 these differences in Kd have profound effects in terms of receptor occupancy. It is worth remembering that the Kd or dissociation constant reflects the concentration of a ligand at which half of the receptor is occupied. Our simple simulation shows that at a low concentration (in the nM range) the high affinity receptors will be occupied at ca. 50% whereas ER binding will be marginal (ca. 10%). In contrast at higher concentrations (mM

range) the occupancy of both receptors will be very high (99%). Note that for this simulation we have taken the much higher value for ER Kd suggesting that for ERα which have apparently and even lower affinity the difference in receptor occupancy will be even higher. Of course such a simulation does not take into account the complexity of the *in vivo* situation: the existence (or not) of endogenous ligands that have to be displaced, the accumulation in some organs of the active molecule, the metabolism of the compounds, the differential expression of the receptors and of the transcriptional co-regulators that mediate the transcriptional receptor effects will all have an important role in regulating receptor occupancy and/or translating the occupancy in a biological activity. Nevertheless our point remains: one should seriously take into account the receptors to explain BPA action especially at low doses. This is particularly true of course for the functions in which these receptors are particularly important. As mentioned above this may be the case for the described metabolic effects of BPA as well as for its effects on immune and nervous systems.

The identification of these receptors must also be taken into account for the question of BPA substitution. Indeed BPA has been banned (or will be) in many countries for products in contact with infants (e.g. baby bottles) and/or general food products. Therefore a huge effort is currently done to identify alternative molecules that will exhibit the same interesting chemical properties (polymerization etc...) but no EDC activity [151]. Up to now, as ERs are considered as the main target of BPA most of these alternatives are only tested for their ability to bind and activate ERs. There is effectively excellent read out, for example based on detailed structural studies, allowing to test these molecules [132, 152]. The identification of GPER and ERRγ as *in vivo* relevant BPA receptors suggests that these molecules should also be taken into account to select inactive BPA alternatives.

A Network of Connected Receptors

There are other possible receptors either members of the nuclear receptor superfamily (listed above) or other transcription factors such as AhR that may also participate to the response. It is clear that it will be very difficult to delineate the precise role of each receptor in the action of BPA and we are clearly facing here a major difficulty. Two factors will render the mechanistical understanding of BPA action very difficult: (i) many of the receptors that are considered as BPA targets are extremely important developmental or physiological regulators and therefore blocking their activity can have by itself major consequences and in some cases (e.g. ERRγ) will be lethal or will seriously impact the target tissue. This implies that the effect of the inactivation of the receptor may preclude the study of BPA effect in its absence. This was the difficulty faced by Tohme et al., since in the absence of either ERRγ-A or ERRγ-B the otic vesicle was severely affected, rendering impossible to study BPA action in this context [117]. It has been necessary to study BPA action in a context in which the expression of the ERRγs was reduced but not totally eliminated and to combine this study with the analysis of a gain-of-function experiment, namely the overexpression of the receptor. Such strategies, plus the use of organ-specific knock-out will be necessary to understand the role of BPA. It will also be important to publish negative results, that is situations in which the inactivation of a given receptor induce no change in BPA action since this can bring useful information. (ii) these receptors are forming a network of connected genes.

For example ERs and ERRs bind on a partially redundant set of response elements (EREs for ERs, EREs and ERREs for ERRs) and therefore can act in a synergistic or antagonistic manner. In addition these receptors are regulating the expression or the activity of others [75]. For instance, ERR have been shown to be a transcriptional activator of the human thyroid hormone receptor a gene [153]. Similarly, there are many deep connections between ER and AhR signalling [154]. This means that deleting one receptor can have an effect on the expression of the other and this again will complicate the determination of what is the primary targeted receptor.

In conclusion, it appears that receptors not related to endocrine function per se (such as orphan receptors currently devoid of ligand) are likely mediators of BPA actions. In this regard, one might question whether other EDCs solely suspected of acting via classical liganded receptors such as ER and AR due to limited data might not actually also impact such kind of orphan receptors. Indeed when considering Table 1 that lists the known molecules able to act on ERRs we can see several molecules that are often discussed in the EDC field such as DES, Chordane or Toxaphene. We may therefore have only touched the top of the iceberg.

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